Identification of Bacteria Harboring Transglutaminase Gene and Its Cloning and Expression in *Escherichia coli*

Suwannee Khunthongpan

A Thesis Submitted in Fulfillment of Requirements for the Degree of

Doctor of Philosophy in Biotechnology

Prince of Songkla University

2018

Identification of Bacteria Harboring Transglutaminase Gene and Its Cloning and Expression in *Escherichia coli*

Suwannee Khunthongpan

A Thesis Submitted in Fulfillment of Requirements for the Degree of

Doctor of Philosophy in Biotechnology

Prince of Songkla University

2018

Thesis Title	Identification of Bacteria Harboring Transglutaminase Gene and Its Cloning and Expression in *Escherichia coli*
Author	Miss Suwannee Khunthongpan
Major Program	Biotechnology

Major Advisor

……
(Asst. Prof. Dr. Punnanee Sumpavapol)

Co-advisor

……
(Prof. Dr. Aran H-Kittikun)

Examining Committee:

.....Chairperson
(Assoc. Prof. Dr. Wanna Choorit)

…….Committee
(Asst. Prof. Dr. Punnanee Sumpavapol)

……Committee
(Prof. Dr. Aran H-Kittikun)

……Committee
(Assoc. Prof. Dr. Tipparat Hongpattarakere)

……Committee
(Asst. Prof. Dr. Apichat Upaichit)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Biotechnology.

……
(Prof. Dr. Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…….....Signature
(Asst. Prof. Dr. Punnanee Sumpavapol)
Major Advisor

……...Signature
(Prof. Dr. Aran H-Kittikun)
Co-advisor

……...Signature
(Miss Suwannee Khunthongpan)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……...Signature
(Miss Suwannee Khunthongpan)
Candidate

ชื่อวิทยานิพนธ์ การจำแนกแบคทีเรียที่มียีนทรานส์กลูตามิเนส การโคลนและการแสดงออกของยีนใน *Escherichia coli*

ผู้เขียน นางสาวสุวรรณี ขุนทองปาน

สาขาวิชา เทคโนโลยีชีวภาพ

ปีการศึกษา 2561

บทคัดย่อ

แบคทีเรียสายพันธุ์ C1112^{T}แยกได้จากน้ำเสียในบ่อบำบัดของโรงงานอาหารทะเลในจังหวัดสงขลา ประเทศไทย เป็นแบคทีเรียแกรมลบ รูปแท่ง ไม่สร้างสปอร์และผลิตเอนไซม์ทรานส์กลูตามิเนส ผลการวิเคราะห์ลำดับวิวัฒนาการของ 16S rRNA gene และ ยีนที่มีการแสดงออกอย่างสม่ำเสมอ (housekeeping genes) จำนวน 5 ยีน ได้แก่ *fus*A, *lep*A, *leu*S, *gyr*B และ *ile*S ซึ่งเกี่ยวข้องกับการสังเคราะห์ Elongation factor G, GTP binding elongation factor, Leucyl-tRNA synthase, DNA gyrase, B subunit และ Isoleucyl-tRNA synthase ตามลำดับ พบว่าแบคทีเรียสายพันธุ์ C1112^{T} มีความเหมือนกับแบคทีเรียในสกุล *Providencia* โดยมีความเหมือนกับ *P. stuartii* DSM 4539^{T} 91.75% และเมื่อทำดีเอ็นเอ-ดีเอ็นเอไฮบริไดเซชั่นกับเชื้อ *P. stuartii* KCTC 2568^{T} พบว่ามีความสัมพันธ์กัน 48.1% แต่คุณสมบัติทางชีวเคมีบางอย่างมีความแตกต่างจากเชื้อแบคทีเรียที่มีสายวิวัฒนาการใกล้เคียงกัน นอกจากนี้แบคทีเรียสายพันธุ์ C1112^{T} มีกรดไขมันที่สำคัญคือ $C_{16:0}$, iso-$C_{15:0}$, $C_{14:0}$ และ $C_{17:0}$ cyclo โดยมี G+C content เท่ากับ 41 mol% การศึกษาลักษณะทางจีโนไทป์และฟีโนไทป์แสดงให้เห็นว่าแบคทีเรียสายพันธุ์ C1112^{T} เป็นแบคทีเรียสายพันธุ์ใหม่ในสกุล *Providencia* โดยให้ชื่อว่า *Providencia thailandensis* และมี type strain คือ C1112^{T} (= KCTC 23281^{T} =NBRC 106720^{T})

แบคทีเรียสายพันธุ์ใหม่ C2361^{T} เป็นแบคทีเรียแกรมลบ รูปแท่ง ไม่สร้างสปอร์ เจริญได้ทั้งที่มีและไม่มีอากาศ และผลิตเอนไซม์ทรานส์กลูตามิเนส ซึ่งคัดแยกได้จากน้ำเสียในบ่อบำบัดของโรงงานอาหารทะเลในจังหวัดสงขลา ประเทศไทย ผลการวิเคราะห์ลำดับวิวัฒนาการลักษณะทางฟีโนไทป์ รวมทั้งสมบัติทางเคมีแสดงให้เห็นว่าเป็นแบคทีเรียในสกุล *Enterobacter* โดยลำดับเบสบน 16S rRNA gene มีความคล้ายกับ *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^{T} และ *Enterobacter cloacae* subsp. *dissolvens* LMG 2683^{T} ที่ 97.5 และ 97.5% ตามลำดับ กรดไขมันที่สำคัญ ได้แก่ $C_{16:0}$, $C_{17:0}$cyclo และ $C_{14:0}$ โดยมี G+C content เท่ากับ 53.0 mol% การศึกษาลักษณะทางฟีโนไทป์ จีโนไทป์ และทางเคมีแสดงให้เห็นว่าแบคทีเรียสายพันธุ์ C2361^{T}

เป็นแบคทีเรียสายพันธุ์ใหม่ในสกุล *Enterobacter* โดยให้ชื่อว่า *Enterobacter siamensis* และมี type strain คือ C2361^{T} (= KCTC 23282^{T} = NBRC 107138^{T})

การแอมพลิไฟล์ยีนทรานส์กลูตามิเนสจากเชื้อ *P. thailandensis* และ *E. siamensis* โดยใช้ไพรเมอร์ที่ออกแบบแบบ specific และ degenerate ในการแอมพลิไฟล์ยีนโดยใช้ดีเอ็นเอพอลิเมอเรส สองชนิด คือ 2× Taq Master Mix (Vivantis) และ Takara *Ex Taq* พบว่าการแอมพลิไฟล์ยีนจากเชื้อ *E. siamensis* โดยใช้ไพรเมอร์ F1R1 และ F2R1 ด้วยดีเอ็นเอพอลิเมอเรส 2× Taq Master Mix ได้แถบดีเอ็นเอขนาด 1,100 bp และ 1,200 bp ตามลำดับ นอกจากนี้เชื้อ *P. thailandensis* ที่ทำการแอมพลิไฟล์ยีนโดยใช้ไพรเมอร์ tgFtgR2 ด้วยดีเอ็นเอพอลิเมอเรส Takara *Ex Taq* พบว่ามีแถบดีเอ็นเอขนาด 1,100 bp จากนั้นได้ทำการเชื่อมต่อดีเอ็นกับพลาสมิด pSSBm97 และศึกษาการแสดงออกของโปรตีนใน *Bacillus megaterium* YYBm1 อย่างไรก็ตามพบว่าไม่สามารถตรวจวัดกิจกรรมของเอนไซม์ทรานส์กลูตามิเนสได้ เนื่องจากเชื้อ *P. thailandensis* และ *E. siamensis* เป็นเชื้อใหม่ซึ่งยังไม่มีข้อมูลของยีนในฐานข้อมูล จึงทำให้ค่อนข้างยากที่จะออกแบบไพรเมอร์เพื่อแอมพลิไฟล์ยีนจากเชื้อทั้งสองชนิด

เชื้อในสกุล *Streptomyces* ซึ่งพบได้มากในดินสามารถผลิตเอนไซม์ทรานส์กลูตามิเนสได้ จากการคัดแยกเชื้อจากดินพบเชื้อ AH6 มีลักษณะเหมือนเชื้อกลุ่ม *Streptomyces* จึงทำการจำแนกเชื้อโดยใช้ 16S rRNA gene พบว่าลำดับเบสของ 16S rRNA มีความเหมือนกับเชื้อ *Streptomyces thermocarboxydus* 99%_โดยให้ชื่อเชื้อ AH6 ว่า *Streptomyces* sp. AH6 จากนั้นทำการโคลนยีนทรานส์กลูตามิเนสจากเชื้อ *Streptomyces* sp. AH6โดยการเชื่อม TGase precursor กับพลาสมิด pET22b แล้วถ่ายโอนดีเอ็นเอลูกผสมไปยัง *Escherichia coli* BL21 (DE3) ผลการอ่านรหัสของดีเอ็นเอลูกผสมนี้พบว่าสามารถแปลงรหัสเป็นกรดอะมิโนได้จำนวน 410 อะมิโน โดยลำดับกรดอะมิโนของเอนไซม์ทรานส์กลูตามิเนสของเชื้อ *Streptomyces* sp. AH6 เหมือนกับลำดับกรดอะมิโนของเชื้อ *S. mobaraensis* NBRC 13476 100% แต่ผลของลำดับนิวคลีโอไทด์มีความต่างกัน 6 นิวคลีโอไทด์ ซึ่งได้แก่ นิวคลีโอไทด์ที่ตำแหน่ง 231, 234, 237, 240, 246 และ 249 โดยดีเอ็นเอลูกผสมที่ได้จากการเชื่อมต่อยีนเรียกว่า pET22b-TG-His6 และมีการแสดงออกของเอนไซม์ทรานส์กลูตามิเนสแบบสารละลาย อยู่ภายในเซลล์ของ *E. coli* จากนั้นทำบริสุทธิ์เอนไซม์ที่ได้พบว่ามีกิจกรรมของเอนไซม์เท่ากับ 3.2 U/ml เอนไซม์สามารถทำงานได้ดีที่ pH และอุณหภูมิเท่ากับ 6.0 และ 40 องศาเซลเซียส ตามลำดับ และถูกยับยั้งการทำงานด้วย Cu^{2+} และ Fe^{2+}

Thesis Title Identification of Bacteria Harboring Transglutaminase Gene and Its Cloning and Expression in *Escherichia coli*

Author Miss Suwannee Khunthongpan

Major Program Biotechnology

Academic Year 2018

ABSTRACT

The bacterial strain C1112^{T} was isolated from seafood processing wastewater collected from a treatment pond of the seafood factory in Songkhla Province, Thailand. It was Gram-negative, rod shape, non-spore former and produced transglutaminase (TGase). Phylogenetic analysis based on concatenated sequences from the 16S rRNA gene and five housekeeping genes, *fus*A, *lep*A, *leu*S, *gyr*B and *ile*S, respectively showed that the strain C1112^{T} belonged to the genus *Providencia*, and shared 91.75% similarity with *P. stuartii* DSM 4539^{T}. DNA-DNA hybridization between the strain C1112^{T} and *P. stuartii* KCTC 2568^{T} was 48.1% relatedness. Moreover, some results from biochemical properties indicated that the strain C1112^{T} was distinguished from the phylogenetically closest relatives. The major fatty acids of strain C1112^{T} were $C_{16:0}$, iso-$C_{15:0}$, $C_{14:0}$ and $C_{17:0}$ cyclo and the DNA G+C content was 41 mol%. Base on the genotypic and phenotypic considerations, it was classified as a novel species of the genus *Providencia* for which the name *Providencia thailandensis* sp. nov. was proposed. The type strain is C1112^{T} (= KCTC 23281^{T} =NBRC 106720^{T}).

A novel strain of *Enterobacter*, C2361^{T}, a Gram-negative, non-spore-forming rod-shaped and facultative anaerobic bacterium with capability to produce the TGase, was isolated from seafood processing wastewater collected from a treatment pond of the seafood factory in Songkhla Province, Thailand. Phylogenetic analysis and phenotypic characteristics, including chemotaxonomic characteristics, showed that the strain was a member of the genus *Enterobacter*. The 16S rRNA gene sequence similarities between the strain C2361^{T} and *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^{T} and *Enterobacter cloacae* subsp. *dissolvens* LMG 2683^{T} were 97.5 and 97.5%, respectively. The major fatty acids were $C_{16:0}$, $C_{17:0}$cyclo and $C_{14:0}$. The DNA G+C content was 53.0 mol%. On the basis of the polyphasic evidences

gathered in this study, it was classified as a novel species of the genus *Enterobacter* for which the name *Enterobacter siamensis* sp. nov. was proposed. The type strain is C2361^T (= KCTC 23282^T = NBRC 107138^T).

TGase genes of *P. thailandensis* and *E. siamensis* were amplified by specific and degenerate primers using 2× Taq Master Mix (Vivantis) and Takara *Ex Taq* as DNA polymerase in PCR reactions. 1,100-bp and 1,200-bp were amplified products from *E. siamensis* genomic DNA, using F1R1 and F2R1 primer with 2× Taq Master. In addition, 1100-bp amplified product from *P. thailandensis* genomic DNA was obtained by using tgFtgR2 primer with Takara *Ex Taq* DNA polymerase and ligated with pSSBm97 and expressed into *B. megaterium* YYBm1. However, TGase activity could not detect from the expressed proteins. Due to *P. thailandensis* and *E. siamensis* are novel species, therefore no information regarding their TGase genes could be acquired from the_database. This makes it quite difficult to design primers for TGase gene amplification from these two bacteria.

Previously, TGase was produced by the genus *Streptomyces* isolated from soil. In this study, *Streptomyces*-like strains were isolated from soil and identified using 16S rRNA gene. The stain AH6 showed 99 % similarity with that of *Streptomyces thermocarboxydus* and named as *Streptomyces* sp. AH6. The TGase gene of *Streptomyces* sp. AH6 was constructed by fusion of TGase precursor and pET22b plasmid. The recombinant DNAs were transformed into *Escherichia coli* BL21 (DE3). The TGase ORF of this gene encoding 410 amino acids. Although the amino acid sequence of theTGase precursor was 100% homologous to that of *S. mobaraensis* NBRC 13476, the nucleotide sequence was different at 6 nucleotide proteins, including the nucleotide 231, 234, 237, 240, 246 and 249. The pET22b-TG-His6 was secreted as a soluble inclusion body in *E. coli* and the activity of partially purified enzyme was 3.2 U/ml. The optimal pH and temperature of this enzyme was 6.0 and 40˚C, respectively. The enzyme was inhibited by Cu^{2+} and Fe^{2}.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Asst. Prof. Dr. Punnannee Sumpavapol and Prof. Dr. Aran H-Kittikun for their excellent supervision, patience and encouragement throughout the course of my study.

I would like to express my sincere thanks to Prof. Yasuhisa Asano for suggestion and support in the experiments and Asst. Prof. Daisuke Matsui for guiding in gene cloning techniques when I was at Toyama Prefectural University, Japan.

I would also like to thank Dr. Suwat saengkerdsub, Dr. Uschara Thumarat and Dr. Wasana Suyotha for supporting plasmid and guiding in gene cloning techniques.

I would also like to thank all members of my committee including Asst. Prof. Dr. Apichat Upaichit, Assoc. Prof. Dr.Tipparat Hongpattarakere and Assoc. Prof. Dr. Wanna Choorit for their advices and suggestion.

I would like to thank the Graduate School of Prince of Songkla University, Thailand, the National Research University Project of Thailand's Office of the Higher Education Commission and the Japan Student Services Organization (JASSO) for the financial support all of this research.

I am very grateful to the members of the Enzyme Technology Laboratory and Food Safety Laboratory, Faculty of Agro-Industry, Prince of Songkla University who give me their help and support during my study.

Finally, I would like to express my deepest gratitude to my family and all my dear friends for their moral support, understanding, inspiration, and encouragement throughout the program.

Suwannee Khunthongpan

CONTENTS

CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

FIGURE **Page**

ABBREVIATIONS AND SYMBOLS

C-terminal	=	Carboxyl terminal
EDTA	=	Ethylenediaminetetraacetic acid
EGTA	=	Ethylene glycol-bis (2-aminoethyl ether) *N*,*N*,*N'N*-tetraacetic acid
g	=	Gram
$\times g$	=	Relative centrifugal force
h	=	Hour
kDa	=	Kilodalton
KPB	=	Potassium Phosphate Buffer
M	=	Molar
mM	=	Millimolar
Mw	=	Molecular weight
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
µl	=	Microliter
µm	=	Micrometer
µmole	=	Micromole
NCBI	=	The National Center for Biotechnology Information
NEM	=	*N'*-Ethylmaleimide
N-terminal	=	Amino terminal
nm	=	Nanometer
PMSF	=	Phenylmethyl sulfonyl fluoride
%	=	Percent
Q	=	Glutamine
R	=	Arginine
rpm	=	Revolutions per minute
SDS	=	Sodium dodecyl sulfate

ABBREVIATIONS AND SYMBOLS (Continued)

SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	=	Trichloroacetic acid
TGase	=	Transglutaminase
U	=	Unit activity

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Transglutaminases (TGases, R-glutaminyl peptide: amine γ-glutaminyl transferase, EC 2.3.2.13) are thiol enzymes that catalyze protein reticulation by introducing isopeptide bonds (ε-(γ-glutaminyl) lysine bonds) (Folk and Finnlayson, 1977). TGases are widely distributed in various microorganisms (Ando *et al*., 1989; Klein *et al*., 1992; Suzuki *et al*., 2000), mollusks (Nazawa *et al*., 2005), fish (Ha and Iuchi, 1997), guinea pig (Folk and Cole, 1966), birds (Suzuki *et al*., 1995) and plants (Mea *et al*., 2004; Villalobos *et al*., 2004). TGases are used in many food production processes to improve the properties of food materials (Ichinose *et al*., 1990) such as texture, stability, and water binding capacity (Yokoyama *et al*., 2004; Dube *et al*., 2007).

Microbial TGases have been reported to produce mainly by the genus *Streptomyces* (Duran *et al*., 1998; Taguchi *et al*., 2002; Lin *et al*., 2006; Marx *et al*., 2008; Ando *et al*., 1989; Wu *et al*., 1996; Yokoyama *et al*., 2004). However, *Bacillus* (Kobayashi *et al*., 1998), *Enterobacter* sp. C2361 and *Providencia* sp. C1112 (H-Kittikun *et al*., 2012) also have been reported to produce the TGase enzyme. The food industries widely use microbial TGases due to the microbial TGases can be mass-produced by conventional fermentation at low cost (Yurimoto *et al*., 2004). The search for microbial TGases from other bacteria could bring new activity profiles with desired properties. In addition, the genetic engineering has been applied to increase the level of TGase production. Moreover, the method of gene cloning could provide opportunity to study the structure and function of gene in detail. While, the expression of gene in *Escherichia coli* is a powerful tool for elucidating protein structure and function relationships and for modifying proteins to improve or alter their characteristics. Then microbial TGase gene from genus *Streptomyces* and *Bacillus* were cloned and expressed to know about their gene sequences and develop a more efficient system for industrial production (Liu *et al*., 2006). At present, *Corynebacterium glutamicum* (Itaya and Kikuchi, 2008), *Candida boidinii* (Yurimoto

et al., 2004) and *Streptomyces* spp. (Lin *et al*., 2006; Liu *et al*., 2006; Noda *et al*., 2010) have been used as host for recombinant TGase expression.

Thus, the main objectives of this research are to isolate TGase-producing bacteria and to clone and express its TGases gene in *E. coli*. Then, the expressed TGase enzyme was partially purified and characterized.

Literature Review

1. Transglutaminases

Transglutaminase catalyses an acyl-transfer reaction between the γ-carboxyamide group of glutamine residue (acyl donors) and a variety of primary amines (acyl aceptors), including the ε-amino group of lysine residue in certain proteins. In the absence of amine substrates, TGases catalyses the deamidation of glutamine residues which water molecules are used as acyl acceptors. TGases can modify proteins by means of amine incorporation, crosslinking and deamidation (Figure 1.1) (Motoki and Seguro, 1998).

TGases are widely distributed in various organisms such as human, bacteria, nematodes, yeasts, alagae, plants and lower vertebrates (Santos and Torné, 2009; Li *et al.*, 2013). TGases from animal tissue, plants and some invertebrates require Ca^{2+} ion for expression of their enzymatic activities (Fredrich and Aszodi, 1992). The functional role of Ca^{2+} is to induce enzyme conformational changes which are responsible for substrate-binding and catalysis (Lorand and Graham, 2003; Tokunaga *et al*., 1993; Greenberg *et al*., 1991; Aeschlimann and Paulsson, 1994). Guinea-pig liver has been the only source of a commercial TGases for decades. The scarce source and the complicated separation and purification process for obtaining tissue TGases have resulted in extremely high price of the enzyme, about USD 80 per activity unit (Meiying *et al*., 2002).

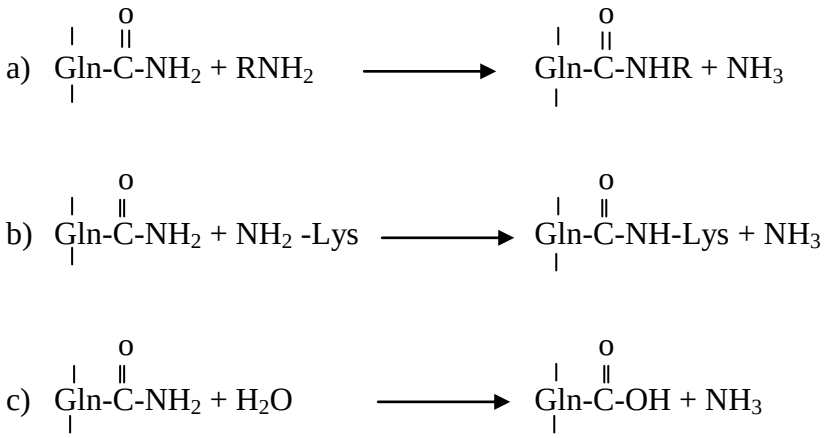


Figure 1.1 Reactions catalyzed by TGases: (a) acyl transfer reaction; (b) crosslinking reaction between glutamine and lysine residues; (c) deamidation reaction.
Source: Motoki and Seguro (1998)

TGases are commonly found inside and outside of a cell. Animal and plant TGases demonstrate catalytic activity and biochemical properties similar to those of microbial TGases, even though it has a lack of homology in the amino acid composition (Luciano and Arntfield, 2012). Animal TGases are involved in spermatogenesis and blood coagulation. While many plant TGases play a role in plants processes of growth and development (Samelak *et al*., 2010). The application of isolated TGases from microbial source has allowed for simplification of certain processes and provided energy and economical savings. Many researchers have been studied gene transfer and expression of genes, which gave rise to TGase production. The transfer of genes to expression systems such as *E. coli*, *S. lividans* and *C. glutamicum* have remarkably increased production efficiency (Kieliszek and Misiewicz, 2014).

2. Bacterial TGases

2.1 TGase-producing bacteria

In 1989, Ando and co-worker reported that strains from the genus *Streptomyces* had the ability to produce TGases as determined by using the hydroxamate assay. These microorganisms which screened from several thousand microorganisms excreted the TGases enzyme, and one of them classified as a variant

of *S.mobaraensis* (Washizu *et al*., 1994) which produced the highest activity. The enzyme from microorganisms was named microbial TGases. Since then, efforts have been made to obtain microbial TGases not only from *Streptomyces* spp. but also from other genus such as *Bacillus* (Barros *et al*., 2003). Microbial TGases is calcium ion-independent and many functional studies demonstrated its industrial application (Kuraishi *et al*., 1996; Motoki and Seguro, 1998; Sakamoto *et al*., 1993; Yokoyama *et al*., 2004). Examples of TGases found in various microorganisms are listed in Table 1.1.

2.1.1 *Streptomycetaceae*

Streptomyces is the largest genus of Actinobacteria and the type genus of the family *Streptomycetaceae* (Hong *et al*., 2009). The *Streptomycetes* are Gram-positive, and the genomes have high GC content. They were most found in soil and decomposed vegetation. *Streptomycetes* are characterised by a complex secondary metabolism. The majority of *Streptomycetes* produce spores and dominant features of their spores is earthy odor which results from the production of a volatile metabolite, geosmin.

The genus *Streptomyces*

Streptomyces is the type genus of the family Streptomycetaceae and currently number of these species were 823 species (Yu *et al*., 2018). The genus *Streptomyces* are Gram-positive bacteria, filamentous and most found in soil. The spores and filaments are very small usually 1 μm or less in diameter (Willemse *et al*.,2011). The *Streptomyces* have high GC content as 69-78%. (Kavitha *et al*., 2010). The strain in genus *Streptomyces* that produce TGase are list in Table 1.1.

Streptomyces thermocarboxydus

Streptomyces thermocarboxydus is moderately thermophilic and carboxydotrophic. It is aerobic, Gram-positive, moderately thermophilic, facultatively chemolithotrophic actinomycete with extensively branched substrate and aerial mycelia (Kim *et al*, 1998). The type strain is *S. thermocarboxydus* AT37.

Streptomyces platensis

Streptomyces platensis strain YK-2 was first isolated from a forest soil sample in Korea and showed a G+C content of 72.7 mol%, contained *meso*-A_2pm as the cell-wall amino acid, and possessed menaquinone MK-9 (H6) and menaquinone

Table 1.1 Transglutaminases producing microorganisms.

Microorganisms	References
Bacteria	
Gram positive	
Bacillus circulans, B. subtilis	Kobayashi *et al*. (1996 and 1998); Suzuki *et al*. (2000); Barros *et al*. (2003); Souza *et al*. (2006)
Streptomyces cinnamoneum, S. fradiae, S. hygroscopicus, S. mobaraensis, S. netropsis, S. platensis, S. baldaccii, S. cinnamoneum, S. ladakanum and *Streptomyces* sp.	Taguchi *et al*. (2002); Date *et al*. (2004); Yurimoto *et al*. (2004); Liu *et al*. (2006 and 2007); Lin *et al*. (2006 and 2007); Cui *et al*. (2006, 2007and 2008); Marx *et al*. (2008); Yu *et al*. (2008); Macedo *et al*. (2010, 2011); Yokoyama *et al*. (2010); Ando *et al*. (1989); Washizu *et al*. (1994); Zhu *et al*. (1996); Duran *et al*. (1998); Negus (2001); Meiying *et al*. (2002); Taguchi *et al*. (2002); Kikuchi *et al*. (2003); Lu *et al*. (2003); Lin *et al*. (2004); Luis *et al*. (2004); Yan *et al*. (2005); Noda *et al*. (2012)
Gram negative	
Enterobacter sp. C2361	H-Kittikun *et al*. (2012); Bourneow *et al*. (2012a)
Providencia sp. C1112	H-Kittikun *et al*. (2012); Bourneow *et al*. (2012a)
Pseudomonas putida	Bech *et al*., 2000
Corynebacterium glutamicum	Date *et al*. (2004)

MK-9 (H8) at a ratio of 6:4. The chemotaxonomic analysis, as well as phylogenetic analysis based on the 16S rDNA sequence, identified the isolate as a member of *S. platensis* (Yeo *et al*., 2009).

Biological mechanism of TGase synthesis from *Streptomyces*

Pasternack *et al*. (1998) revealed that *Streptomyces* was secreted TGase as a zymogen (pro-TGase) and could be activated by several exogenous proteases, such as bovine trypsin, intestinal chymotrypsin or dispase from *B. polymyxa*. A metalloprotease from *S. mobaraensis* is an endogenous TGase-activating protease (Zotzel *et al.*, 2003). In 2008, Zhang *et al.* found that in *S. hygroscopicus,* not only endogenous metalloprotease but also endogenous serine protease is involved in TGase activation. In additional, this research indicated that pro-TGase of *Streptomyces* appear to have a conserved amino acid sequence preceding the N-terminal of TGase, which contains cleavage sites for both serine protease and metalloprotease, indicating activation of pro-TGase is not a specific process. Recently, 4 different wild strains of *Streptomyces* have been studies for TGase production. These strains were shown in Table 1.2.

Table 1.2 Microbial TGase production by wild strains of *Streptomyces* sp.

Year	Strain	Yield (U/ml)	References
1989	*S. mobaraensis*	2	Ando *et al*., 1989
2004	*S. ladakanum*	0.348	Tellez-Luis *et al*., 2004
2007	*Streptomyces* sp.	1.4	Macedoa *et al*., 2007
2007	*S. hygroscopicus*	5.04	Cheng *et al*., 2007

2.1.2 *Enterobacteriaceae*

The *Enterobacteriaceae* is a bacteria in phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales and family *Enterobacteriaceae* which is large, heterogeneous group of Gram-negative, facultative anaerobic, rod-shaped bacteria that do not form endospores with catalase-positive and oxidase-negative (Brenner, 1984). Members of the family are distributed worldwide, with some being saprophytes and others being parasites of plants and animals. Many species of this family are of considerable economic importance due to their pathogenic effects on

agriculture and livestock (Janda and Abbott, 2006). Currently, there are 52 genera described in this family, which include *Arsenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Calymmatobacterium, Cedecea, Citrobacter, Cosenzaea, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Gibbsiella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Levinea, Lonsdalea, Mangrovibacter, Moellerella , Morganella, Obesumbacterium, Pantoea, Pectobacterium, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Shimwellia, Sodalis, Tatumella, Thorsellia, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia* and *Yokenella* (http://www.bacterio.cict.fr/e/ enterobacter.html).

The genus *Enterobacter*

The genus *Enterobacter* are gram-negative bacteria, rod-shaped, most of which are motile, peritrichous flagella, oxidase-negative and have relatively simple growth requirements (Barrow and Feltham, 1993; Stephan *et al*., 2008). Features such as motility, as well as certain biochemical properties, including the ability to synthesize an enzyme known as ornithine decarboxylase, are used to distinguish *Enterobacter* from the very similar and closely related *Klebsiella* bacteria. *Enterobacter cloacae* being the type species of this genus. *E. cloacae* is Gram-negative, rod-shaped, facultative and peritrichous flagella. Biochemical reactions of this strain showed that it was positive of indole, methyl red test but negative for Voges-Proskauer test. The decarboxylase test pattern was quite characteristic: negative, positive, and positive for lysine, arginine, and ornithine, respectively. This strain fermented arabinose and rhamnose with the production of gas (Hormaeche and Edwards, 1960; Washington *et al*., 1969). In present, the genus *Enterobacter* has 25 species and 2 subspecies with validly published names (http://www.bacterio.cict. fr/e/enterobacter. html). Optimum temperature for growth is 30˚C (Sanders *et al*., 1997). Most clinical strains grow at 37˚C and environmental strains give erratic biochemical reactions at 37˚C. The DNA G+C mol% of the other members of genus *Enterobacter* are fall within a range of 52-60 mol% (Saitou *et al*., 1987; Duran *et al*., 1998; Mea *et al*., 2004; Yokoyama *et al*., 2004).

In addition, *Enterobacter* sp. strain C2361 was isolated from wastewater ponds of a surimi processing factory in Songkhla Province, Thailand (H-Kittikun *et al*., 2012) and it was rapidly produced TGase in SPY medium (2% of starch, 2% of peptone, 0.5% of yeast extract, 0.2% of $MgSO_4$, 0.2% of K_2HPO_4, 0.2% of KH_2PO_4 and pH 7.0), at 37˚C with shaking at 150 rpm, which could be reached to the highest activity of 0.87 U/ml at 36 h (Bourneow, 2011).

The genus *Providencia*

The members of the genus *Providencia* are all facultative anaerobes and motile by peritrichous flagella (Manos and Belasb, 2006). However, they do not exhibit cellular differentiation and swarming behavior. Urease production is not characteristic of all *Providencia* species, with only *P. rettgeri* strains producing urease (Brenner *et al*., 1978). The genus *Providencia* have been isolated from humans, insects, many other vertebrate and invertebrate animals (Penner and Hennessy, 1979; Muller *et al*., 1986; Yoh *et al*., 2005; Somvanshi *et al*., 2006). The genus *Providencia* consists of eight species as *P. stuartii*, *P. sneebia*, *P. rettgeri*, *P. rustigianii*, *P. heimbachae*, *P. burhodogranariea*, *P. alcalifaciens*, *P. vermicola* (O'Hara *et al*., 2000; Somvanshi *et al*., 2006). The optimal growth temperature of *Providencia* sp. is 37˚C (Juneja and Lazzaro, 2009). The G+C content of the genus *Providencia* is 39.6-43.0 mol% (Owen *et al*., 1987).

In 2012, *Providencia* sp. strain C1112 was isolated from wastewater ponds of a surimi processing factory in Songkhla Province, Thailand (H-Kittikun *et al*., 2012). The TGase of this strain was rapidly produced in SPY medium at 37˚C with shaking at 150 rpm. The highest activity was 0.82 U/ml at 12 h cultivation (Bourneow, 2011).

2.2 Factors affecting on microbial TGase production

The media composition used to produce microbial TGase from microorganisms have been almost the same in every work published (the basal medium for cultures contained: 2% of starch, 2% of peptone, 0.5% of yeast extract, 0.2% of $MgSO_4$, 0.2% of K_2HPO_4, 0.2% of KH_2PO_4 and pH 7.0; 0.2% of peptone; 0.2% K_2HPO_4; 0.1% MgSO4.7H2O; 0.5% of glucose and pH 7.0) (Ando *et al*., 1989; Zheng *et al*., 2001, 2002; Yan *et al*., 2005; Bahrim *et al*., 2010). The results of the effects of the carbon and nitrogen sources on the microbial TGase activity produced

by *Streptomyces* sp. P20 was reported by Macedo *et al*. (2007). The most promising nitrogen sources was peptone, whereas the carbon sources were obtained with a mixture of potato starch, glucose and with maltodextrin.

2.2.1 Nitrogen source

Zhu *et al*. (1996) investigated a batch fermentation for the production of the microbial TGase from *S. mobaraense.* It was found that amino acids play an important role in the synthesis of cell mass and microbial TGase. Peptone is a favorable nitrogen source for optimal growth and microbial TGase production. In a fed-batch system, glutamine is a major nitrogen sources, however, it was also responsible for the production of ammonium, toxic by-products. Therefore, the strategy should be not only to develop a medium that provides these main materials, but also to control the culture environment to eliminate the production of ammonium (Xie and Wang, 1994). During the stationary phase, the supply of nitrogen was provided by the remaining amino acids and by ammonium sulphate and yeast extract in the feed. Microbial TGase production from *S. mobaraense* was stopped at 34 h, with the activity of 1.8 U/ml (Zhu *et al*., 1996). The strain *Streptomyces* MIUG 13P produced the highest yield of TGase after 10 days of submerged fermentation in the basal medium using peptone as nitrogen source (Bahrim *et al*., 2010). Aidaroos *et al*. (2011) reported the profile of *S. hygroscopicus WSH03-01* grown with ammonium sulphate as nitrogen source was reached stationary phase at 42 h and gradually microbial TGase yield was increasing till 3.2 U/ml at 60 h.

2.2.2 Carbon source

A high microbial TGase activity (0.73 U/ml) was obtained using glycerol as a carbon source and casein on production of TGase by *S. ladakanum* (Tellez-Luis *et al*., 2004). The consumption of carbon source in the cultivation of *S. ladakanum* was different for sugar and glycerol. The mixture of sugar cane molasses and glycerol is an adequate culture medium for the production of microbial TGase by *S. ladakanum*. *Streptomyces* MIUG 13P produced the highest yield of TGase after 10 days of submerged fermentation in the basal medium using potato starch, glucose and soybean powder as carbon source (Bahrim *et al*., 2010).

2.2.3 Cultivating conditions

Temperature is one of the most important environmental factors for cell growth and product formation. The microbial TGase formation rate, activity and microbial TGase productivity in batch culture of *S. mobaraense* vary with medium compositions and environmental parameters, including pH (6.5-7.5), temperature (optimum at 27-35˚C) and dissolved oxygen (Zheng *et al*., 2001, 2002). It is very important to control cell growth and product formation process under appropriate conditions during the microbial TGase fermentation (Yan *et al*., 2005).

2.4 Purification and characteristics of microbial TGase

2.4.1 TGase purification

Suzuki *et al*. (2000) purification and characterization of novel TGase from *B. subtilis* AJ 1307. The enzyme was purified in three steps (hydrophobic interaction chromatography, Sephacryl S200 column and anion-exchange chromatography). The specific activities of the purified TG1 and TG2 were estimated to be 0.047 and 0.051 U/mg, respectively. Microbial TGase stability of *S. hygroscopicus* WSH03-13 was strongly affected by high concentration of ethanol. In addition, higher temperature is another important factor affecting the enzyme stability in the presence of ethanol. The result was observed that microbial TGase was not stable at 50˚C (loss of over 50% after 30 min of incubation) and became more sensitive to heat in the presence of ethanol (Cui *et al*., 2006). During the precipitation step, chilled ethanol was used to precipitate the enzyme proteins. The precipitate formed was immediately dissolved in 20 mM phosphate buffer, pH 6.0 and dialyzed against the same buffer overnight at 4˚C. Under these conditions, ethanol concentration in the enzyme solution was very low and a high yield of enzyme activity could be obtained. About 85% of the enzyme activity was precipitated with 70% ethanol with a two fold increase in the specific activity. The initial effective and efficient purification step was chromatographed on CM-cellulose. Microbial TGase could be eluted between 0.3 and 0.4 M NaCl with a yield of 57.7%, a specific activity of 6.26 U/mg protein and 25-fold purification. Further purification was achieved by subjecting the pooled enzyme fraction from the CM-cellulose column to a Sephadex

G-75 column, which resulted in a 30-fold purification with a yield of 21.1% and a specific activity of 7.6 U/mg protein (Cui *et al*., 2007).

In 2011, the microbial TGases of *Enterobacter* sp. C2361 and *Providencia* sp. C1112 was studies. Microbial TGases from *Enterobacter* sp. C2361 and *Providencia* sp. C1112 were precipitated by 70% ethanol, 70% acetone and 50-80% ammonium sulfate ($(NH_4)_2SO_4$) and the total TGases activity of 74 U/mg , 72 U/mg and 101 U/mg were obtained respectively. The microbial TGases of *Enterobacter* sp. C2361 was purified by using SP-sepharose and sephadex G-100, with 19-fold purification and 15% yield. The TGase of *Providencia* sp. C1112 was also purified by using SP-sepharose and sephadex G-100, with 26-fold purification and 14.8% yield (Bourneow, 2010).

The crude enzyme preparation from *Streptomyces* sp. was purified on a Sephadex G-75 column, which resulted in a 2.3-fold purification, a yield of 48% and a specific activity of 1.92 U/mg. The pooled enzyme fraction from the first column was run through the same Sephadex G-75 column, which resulted in a 5.0-fold purification with a yield of 17.7% and a specific activity of 4.18 U/mg protein (Macedo *et al*., 2011). The TGase of *Streptomyces ladakanum* BCRC 12422 were purified on CM-Sepharose CL-6B and Blue Sepharose Fast flow column. The purified microbial TGase showed molecular mass approximately 39,000 Da on SDS-PAGE and 38,210 Da using mass spectrometer (Tzeng *et al*., 2005).

2.4.2 Effect of pH on activity and stability

The optimum pH for microbial TGase activity was around 5 to 8. However, even at pH 4 or 9, microbial TGases still express some enzymatic activity. Microbial TGases are thus considered to be stable over wide pH range (Motoki and Seguro, 1998). Optimal pH for TGase (TG1 and TG2) of *B. subtilis* AJ 1307 was 8.2 (Suzuki *et al*., 2000). The TGase of *S. hygroscopicus* exhibited optimum activity in a range of pH 6.0 to 7.0 and showed some activity at pH 4.5 or 9. The optimum pH of this enzyme was nearly the same as that from *S. mobaraense* (Ando *et al*., 1989). The activity was found to decrease gradually at alkaline pH, but it decreased rapidly at acidic pH (Cui *et al*., 2007). The optimum pH for TGase activity of *Enterobacter* sp. C2361 was in a range of pH 5.0 to 6.5 and *Providencia* sp. C1112 was in a range of pH 5.0 to 7.0 and showed the highest activity at pH 6.5 (Bourneow, 2011). While the

enzyme of *Streptomyces* sp. exhibited optimum activity in a pH range of 6.0 to 6.5 and was stable from pH 5.0 to 8.0 (Macedo *et al*., 2011).

2.4.3 Effect of temperature on activity and stability

Temperature has an influence on TGase activity and stability. Different microorganisms have different optimal temperature for growth and TGase production. The optimal temperature for TGase activity of *S. mobaraense* is 55˚C and for *S. griseocarneum* and *S. cinnamoneum* were 45˚C (Ando *et al*., 1989; Jiang and Lee, 1992). Wherease the optimal temperature for TGase (TG1 and TG2) activities of *B. subtilis* AJ 1307 was around 60˚C (Suzuki *et al*., 2000). Cui *et al.* (2007) reported the TGaes from *S. hygroscopicus* exhibited optimum activity at 37 to 45˚C (in a range of pH 6.0 to 7.0). While the optimal for TGase activity temperature of *Enterobacter* sp. C2361 was at 37 to 50˚C and of *Providencia* sp. C1112 was at 37 to 45˚C (Bourneow, 2011).

The stability of the enzyme decreases with increased temperature. The TGase of *S. mobaraense* remained 100% activity at 40˚C and 74% activity at 50˚C (Ando *et al*., 1989). Moreover, the activity of *S. hygroscopicus* TGases was not stable above 50˚C (Cui *et al.*, 2007).

2.4.4 Substrate specificity

Most food proteins, such as legume globulin, whete gluten, egg yolk and egg white proteins, actins, myosins, fibrins, milk caseins, α-lactalbumin and β-lactoglobulin, as well as many other albumin, could be crosslinked by microbial TGase. (Motoki and Seguro, 1998).

The substrate specificity of TGases for primary amines was investigated to incorporate various functional groups into proteins and peptides. For the primary amines to be incorporated into benzyloxycarbonyl-L-Gln-Gly (Z-Gln-Gly), they were required to have more than four carbon chains without side chains between the functional groups. These suggested that appropriate primary amines as spacers, various functional groups, carboxyl groups, phosphate groups, saccharides, and so on, can be incorporated into proteins by using TGases (Ohtsuka *et al*., 2000).

2.4.5 Metal ions

Metal ions play essential roles in about one third of enzymes. These ions can modify electron flow in a substrate or enzyme, thus effectively controlling an

enzyme-catalyzed reaction. They can serve to bind and orient substrate with respect to functional groups in the active site, and they can provide a site for redox activity if the metal has several valence states. The enzyme provides an arrangement of side chain functional groups having an appropriate sized hole with the preferred groups on enzyme side chains needed to bind the required metal ion (Glusker *et al*., 1999). The residues that most often bind metal ions are CYS, HIS, GLU and ASP (Auld, 2001) because the atoms of their polar or charged side chains can coordinate metal ions.

TGase from guinea pig liver enzyme requires Ca^{2+} for expression of enzymatic activity. The sensitivity of microbial TGase toward other cations in the absence of reducing agents was investigated (Seguro *et al*., 1996). Cu^{2+}, Zn^{2+}, Pb^{2+} and Li^{+} significantly inhibited microbial TGases. Heavy metals such as Cu^{2+}, Zn^{2+} and Pb^{2+} bind the thiol group of the single cystein residue, this strongly supports the idea that the cystein residue could be part of the active site of microbial TGases (Tsai *et al*., 1996).

Cui *et al.* (2007) reported the TGase from *S. hygroscopicus* was not inhibited by Ca^{2+} and ethylenediaminetetraacetic acid (EDTA). The purified TGase was strongly inactivated by 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), Cu^{2+}, Zn^{2+}, Pb^{2+} and Hg^{2+}. The result suggested that this enzyme could possess a thiol group at the active site.

The purified TGase from *Enterobacter* sp. C2361 and *Providencia* sp. C1112 were moderately inhibited by Mn^{2+}, Hg^{2+}, Ba^{2+}, Cu^{2+} and Zn^{2+} at 10 mM. Co^{2+} and Na^{+} possessed less effect on lowering of TGases activity (Bourneow, 2011).

2.4.6 Enzymatic inhibitors

The binding of an inhibitor can stop a substrate from entering the enzyme's active site or hinder the enzyme from catalyzing its reaction. Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually react with the enzyme by covalent bond formation with key amino acid residues needed for enzymatic activity. In contrast, reversible inhibitors bind non-covalently to the enzyme, the enzyme-substrate complex, or both.

TGases are sensitive to the chelating agents such as ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and EDTA. However, those chelating agents could not inhibit the catalytic activity of microbial TGases. In

addition, many synthetic inhibitors including, iodoacetamide (IAA), dithiothreitol (DTT), N-ethylmaleimide (NEM), phenylmethanesulfonylfluoride (PM) or phenylmethylsulfonyl fluoride (PMSF), p-chloromercuribenzoate (pCMB) could inhibit TGases (Nielsen, 1995). Ando *et al*. (1989) reported that the addition of pCMB, NEM and mono-iodoacetate (MIA) destroyed the sulphydryl group of microbial TGases, resulting in inhibition of microbial TGase activity.

3. Structure of TGase gene

3.1 Comparison of the active site of *TGase*

The mammalian *TGase* had a highly conserved active site region (YGQCWVF) which includes an essencial Cys residue. Aeschilman and Paulsson (1994) used the active site sequence of mammalian *TGase* as probes for a computer search in protein databases. They reported that the catalytic region of various thiol proteases were identified as the most closely homogous domains. In addition to the highly conserved active site region, mammalian TGase require calcium for activation. The amino acid sequence of the putative calcium-binding domain of factor XIIIa is thought to be highly conserved in other mammalian *TGase*. The similarities of conserved sequence suggest that these regions are closely related to the function of mammalian *TGases.*

For *Streptomyces TGase*, there is little similarity in the amino acid sequence of the active site region except for the single Cys residue and there is no sequence homology with the calcium binding domain as microbial TGase catalyses a Ca^{2+}-independent acyl transfer reaction (Kanaji *et al*., 1993). The amino acid sequence of microbial TGase from *S. mobaraense* bears no significant similarity to any other proteins.

The amino acid sequences of mammalian *TGase* and microbial *TGase* from *Streptomyces* shows no overall structureal relationship except in the regions around the Cys residue (YGCVG) (Kanaji *et al*., 1993). *Bacillus TGase* has little overall sequence similarity with microbial *TGase* or mammalian *TGase* except for the Cys residue in the active site (Kobayashi *et al*., 1998). Amino acid sequence in putative active site of TGase from various species are shown in Table 1.3.

3.2 Sequence and structure conservation of the *TGase*

Makarova *et al*. (1999) identified a superfamily of proteins homologous to eukaryotic TGase. Sequence and structure conservation of the *TGase* were analyzed and profiles were generated by the PSI-BLAST Program. During comparative analysis of the archaeal genomes. They identified a family of large multidomain archaeal proteins that encompassed predicted signal peptides and a globular domain (as predicted using the SEG program (Makarova *et al*., 1999; Wootton, 1994; Wootton and Federhen, 1996). The latter domain contains conserved

Table 1.3 Amino acid sequence in putative active site of TGase from various species.

Organism	**Amino acid sequence in putative active site of TGase**
S. mobaraense	WLSYGC*VGVTWVNSGQYPTRN
S. ladakanum	EWLSYGC*VGVTWVNS
S. platensis	QLSYGC*VGVTWVNTGP
B. subtilis	FYAFEC*ATAIVIIYYLALID
Factor XIIIa	VRYGQC*WVFAGVFNTFLRCLG
TG_k-human, TG_k-rat, TG_k-rabbit	VPYGQC*WVFAGVTTTVLRCLG
TG_C-guinea pig, TG_C-human, TG_C-mouse, TG_C-bovine	VKYGQC*WVFAAVACTVLRCLG

The active site of TGase is underlined.

*: Active site Cys residue, TG_k: Keratinocyte TGase, TG_C: Tissue TGase.

Source: Negus (2001); Kanaji *et al*. (1993); Kobayashi *et al.* (1998); Lin *et al*. (2004, 2006).

cysteine, histidine, and aspartate residues, which is reminiscent of the catalytic triad of a variety of thiol hydrolases. Further iterative searches of the nonredundant database (NR) using the PSI-BLAST program (Altschul *et al*., 1997) with a number of different query sequences not only detected homologous protein sequences in bacterial genomes but also demonstrated a statistically significant similarity to animal TGases. All sequences of TGase homologs detectable in the current nonredundant database were identified via transitive PSI-BLAST searches, and a representative set of them was used to generate a multiple alignment with the ALITRE program (Seledtsov *et al*., 1995). The alignment was then manually adjusted on the basis of alignments generated by PSI-BLAST and the structural elements from the crystal structure of factor XIII. Figure 1.2 shows the multiple alignment of the TGase-like superfamily with the secondary structure elements assigned using the factor XIII structure. The sequence conservation in the superfamily of TGase-like enzymes clearly centers around the three (predicted) active residues of the catalytic triad. Motif I contains the catalytic cysteine and encompasses the two strands and an α-helix whereas motifs II and III include the two strands associated with the active histidine and aspartate, respectively. Some of the characteristic features of the animal TGase, namely the tiny residue located two positions upstream of the catalytic cysteine, the aromatic residues two positions downstream from the catalytic histidine and the aromatic residue flanking the catalytic aspartate from the N-terminal side, are well conserved in the microbial proteins.

The alignment was constructed using the ALITRE program and adjusted manually on the basis of the PSI-BLAST search results. The TGase-like domains were clustered by sequence similarity on the basis of a detailed examination of the PSI-BLAST search results obtained, for which portions of the respective proteins that include the core domain together with flanking region were used as queries. Family 1 includes seven distinct functional types of mammalian TGases represented here by the respective human proteins; the other seven families include previously undetected TGase homologs. The numbers between aligned blocks indicate the lengths of variable inserts that are not shown; the numbers at the end of each sequence indicate the distances from the protein termini to the proximal and distal aligned blocks. The shading of conserved residues is according to the

consensus and includes residues conserved in at least 80% of the aligned sequences. The three residues of the catalytic triad are shown in inverse shading (yellow against a dark blue background); the putative alternative catalytic cysteine in Family 2 is shown in white against a light blue background; the putative catalytic serine in an *Aquifex* protein is shown in red (Family 4). The fragments of the papain sequence surrounding the catalytic residues were incorporated in the alignment manually, on the basis of the published structural comparisons (Pedersen *et al*., 1994). In the consensus line, h indicates hydrophobic residues (A, C, F, L, I, M, V, W, Y; yellow background); s indicates small residues (A, C, S, T, D, N, V, G, P; blue background); c indicates charged residues (R, K, E, D, H; brown coloring); separately conserved residues are colored in magenta. PDB record 1fie. The GeneBank gene identifier and name (following an underline) or an abbreviated species names where a gene name is not available are shown at the beginning of each sequence. Gene names that start with AF are from *A. fulgidus*; MJ, *Methanococcus jannaschii*; MTH, *M. thermoautotrophicum*; PH, *Pyrococcus horikoshii*; Rv, *Mycobacterium tuberculosis*; YDL117w and YPL096w are from the yeast *S. cerevisiae* and YEB8 is from the yeast *S. pombe*. Other species abbreviations: Aquifex, *Aquifex aeolicus*; Bacillus, *B. subtilis*; Caenorh, *C. elegans*; Hum, *Homo sapiens*; Rat, *Rattus norvegicus*; Synech, *Synechocystis* sp. 112798_4.2 is human band 4.2 protein (an inactivated transglutaminase); TGLC-P are various human TGase isoenzymes; 1942386_XIII is human blood-clotting factor XIII; and 3249613_MTH_PHAG is the pseudomurein endoisopeptidase from the *Methanobacterium* bacteriophage psiM2. The secondary structure elements extracted from the crystal structure of the human blood-clotting factor XIII (Yee *et al*., 1994) and papain (Schroder *et al*., 1993) are shown underneath the alignment; = = indicates β-strands and @@ indicates α-helix.

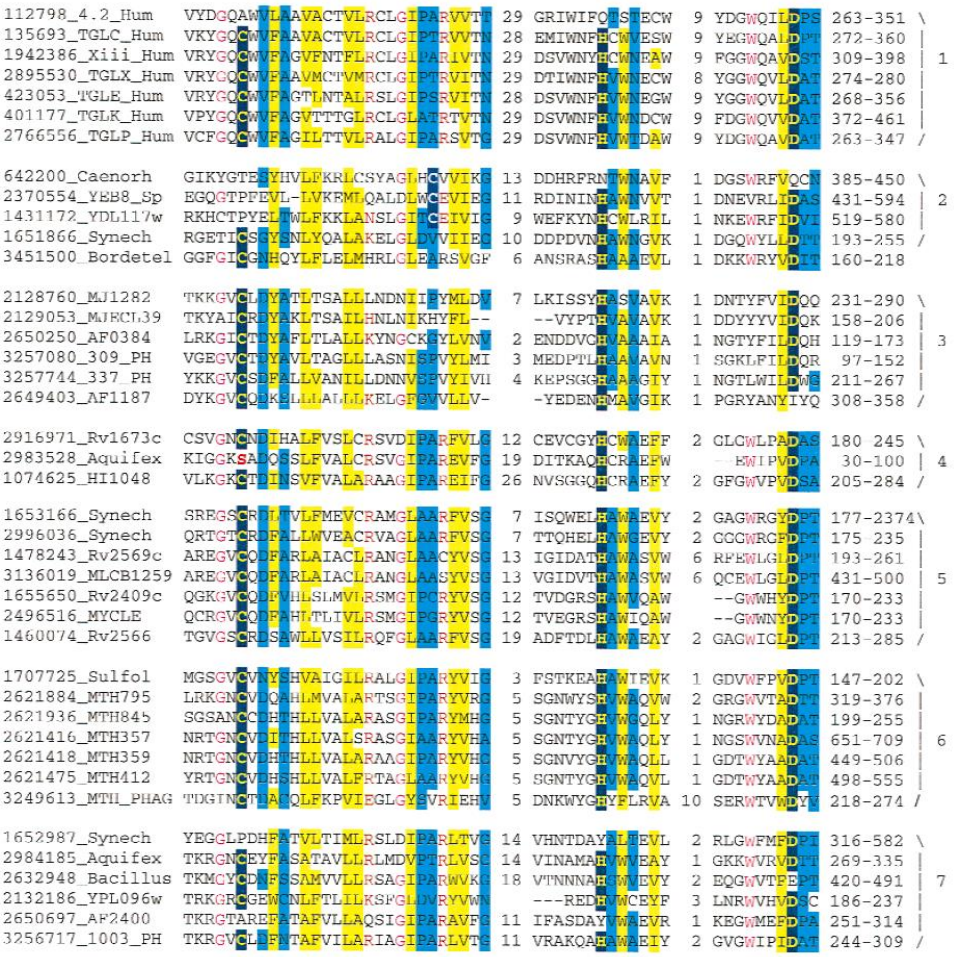


Figure 1.2 Multiple alignment of the conserved core of the TGase-like protein superfamily.

Source: Makarova *et al*. (1999)

3.3 Microbial TGase gene

TGase gene of *Streptomyces* sp. was analyzed. The results showed that an open reading frame (ORF) of *S. cinnamoneum* CBS 683.68 was 1,239 bp which started from an ATG codon at position 195 and terminated at a TGA codon at position 1,239 bp (Duran *et al*., 1998). Taguchi *et al*. (2002) reported the microbial pro-TGase gene from *S. cinnamoneum.* The result showed that an ORF of 1,251 bp, which started from an codon at position 151 and terminated at a TGA codon at position 1,401 and microbial pro-TGase gene was assumed to synthesis as a protein precursor of 416 amino acid residues, which included a 32 amino acid signal-peptide and a 54 amino acid pro-peptide, yielding a mature TGase of 330 amino acid residues in the secretion

process. The amino acid sequence of TGase gene from *S. cinnamoneum* IFO 12852 was slightly different from that of TGase gene from *S. cinnamoneum* CBS683.68. TGase gene from *S. ladakanum* revealed 1,230 bp and it encoded a protein of 410 amino acids. The gene starts from an ATG codon at position 683 and terminates in a TGA codon at position 1,913 (Lin *et al*., 2004). Next, the nucleotide sequence of *TGase* from *S. platensis* M5218 revealed ORF of 1,254 bp, it encodes a protein of 418 amino acids. The gene starts from an ATG codon at position 1119 and terminates in a TAA codon at position 2,373 (Lin *et al*., 2006). Liu *et al*. (2006) analyzed the sequences of *S. platensis*, the ORF of the TGase gene was 1,242 bp, encoding 413 amino acids. TGase gene consists of 331 amino acids with the previous 82 amino acids corresponding to the prepro-region.

Previous study, reported that the initiation codon of TGase gene was preceded by the nucleotide sequence, which may serve as the Shine-Dalgarno sequence. Ribosome-binding sequence of TGase genes were analyzed from *Streptomyces* sp. Duran *et al*. (1998) reported that ribosome-binding sequence of *S. cinnamoneum* CBS 683.68 was AAGGA. Lin *et al*., 2006 and Bae *et al*., 2012 determined ribosome-binding sequence of *S. platensis* M5218 and *S. platensis* YK-2. The results were found that ribosome-binding sequence as AGGGAG. Liu *et al*. (2006) reported ribosome-binding sequence of *S. fradiae* was AAGGGAG and Yu *et al*. (2008) analyzed ribosome-binding sequence of *S. netropsis*. The result showed sequence was AGGGA. Additional, the N-terminal amino acid sequence of *S. cinnamoneum* CBS 683.68 were determined by protein sequencing was found within the deduced amino acid sequence from position 82 to 112 (Duran *et al*., 1998) and Liu *et al*. (2006) analyzed N-terminal amino acid sequence of *S. fradiae* determined by protein sequencing was found within the deduced amino acid sequence from position 83 to 90. In 2008, Yu *et al*. purified *S. netropsis* TGase was then subjected to N-terminal amino acid sequence analysis. The first nine amino acids determined by the Edman degradation method have the sequence as follows: LVDDRETPP.

4. Gene cloning and expression of microbial *TGase*

In order to improve the TGase production, researchers have tried to increase TGase yields using genetically modified strains with molecular techniques. There are five strategies used for increase the expression and solubility of over expression protein (1) changing the vector, (2) changing the host, (3) changing the

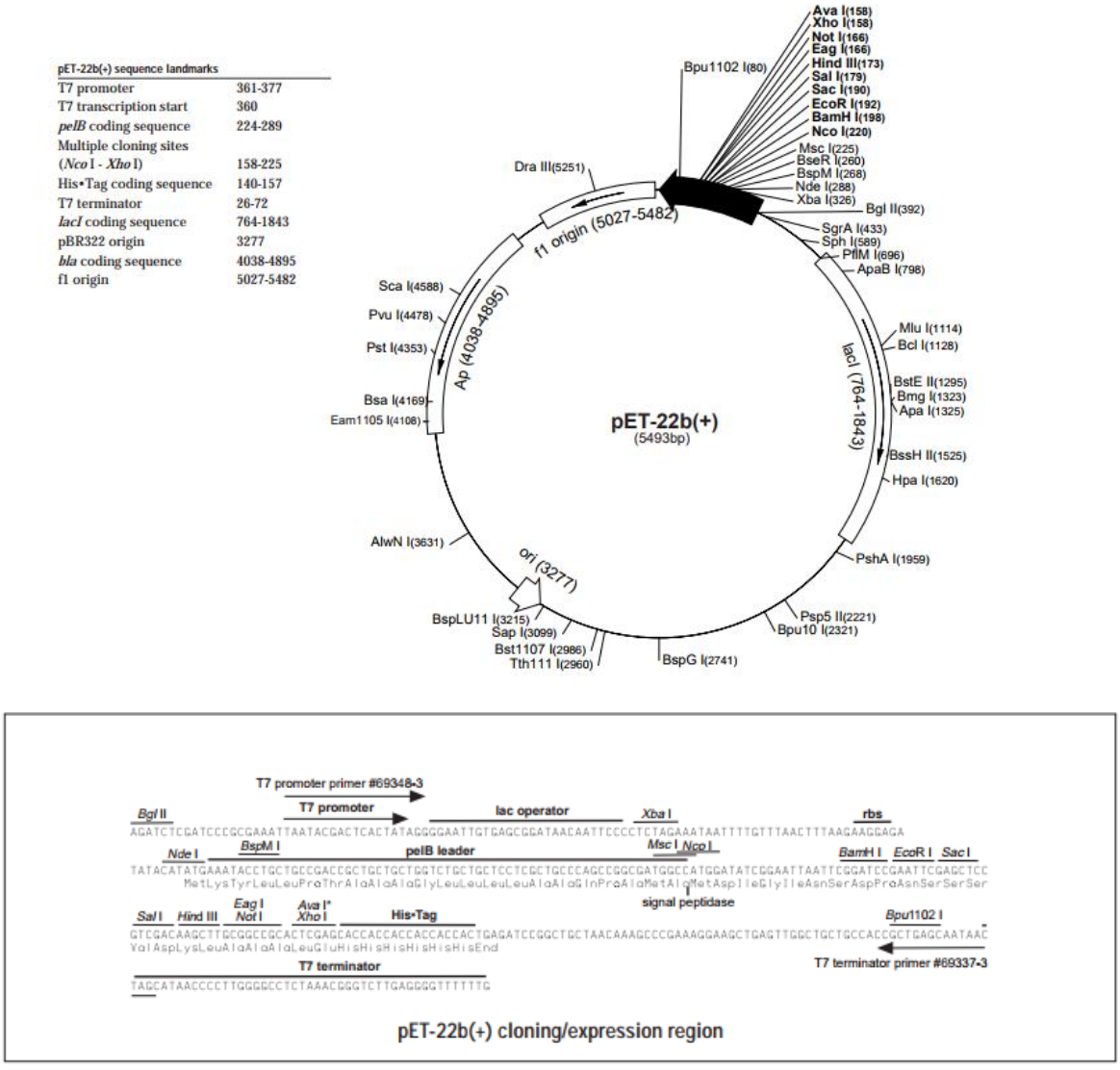


Figure 1.3 Map of pET-22b(+) Vector.

Source: Novagen (2003)

culture parameters of the recombinant host strain, (4) co-expression of other genes and (5) changing the gene sequences (Gopal and Kumar, 2013).

The pET22b+ vector (Figure 1.3) is always the first choice to improve the production of TGase by genetically modified strains (Liu *et al*., 2011a; 2011b) due to this vector has 6-Histidine tag or poly-His-tag (His-tag). His-tag was fused to N- or C-terminus of the vectors for production of recombinant proteins. Expressed His-tagged proteins were easy to purified and detected.

Escherichia coli is the most commonly used as a host strain, which expressed high yield of recombinant protein (Robichon, *et al*., 2011). Accordingly, *E. coli* B strain is popular for recombinant protein expression. The DE3 strain contained the λDE3 lysogen that carried the gene for T7 RNA polymerase under control of the *lac*UV5 promoter. IPTG is used to induce expression of the T7 RNA polymerase in order to express recombinant genes cloned downstream of a T7 promoter. BL21(DE3) is suitable for expression from a T7 promoter.

The promoter is an important target for increasing recombinant protein production because of its role in controlling the transcription initiation of associated genes. The promoter consists of DNA sequences crucial for recognition by the RNA polymerase. In *E. coli* these sequences are compromised by the core sequences, usually centered around positions -10 and -35, an extended -10 element and occasionally a up element (Browning and Busby, 2004). Consensus sequences for the σ70 -recognized -35 and -10 sequences in *E. coli* are shown in Table 1.4. Transcription initiation requires the core RNA polymerase (consisting of the subunits ββ'α2ω) and a specific σ initiation factors to form the holoenzyme. The holoenzyme will bind promoters differentially as specified by its σ factor. *E. coli* has seven different σ factors. The predominant σ70 is responsible for controlling transcription of the housekeeping genes whereas the six alternative factors are required for transcription of smaller subsets of promoters (Gruber and Gross 2003). The association between the holoenzyme and the promoter is stabilized by the differentiating contacts between the σ factor and the core sequences of the target promoter and the extended -10 element, and contacts between the α subunit and the UP element. Subsequent unwinding of the duplex DNA from approximately the -10 to the +2 position facilitates contact between the template

Table 1.4 DNA sequences of some promoters used in *Escherichia coli* expression vectors recognized by the housekeeping sigma factor σ70.

Promoter	-35 region	Spacer	-10 region
lac	TTtACA	18 bp	TATgtT
*lac*UV5	TTtACA	18 bp	TATAAT
trp	TTGACA	17 bp	TtaAcT
tac	TTGACA	17 bp	TATAAT
trc	TTGACA	18 bp	TATAAT
λP_L	TTGACA	17 bp	gATAcT
λP_R	TTGACt	17 bp	gATAAT
lacI	gcGcaA	17 bp	cATgAT
$lacI^q$	gTGcaA	17 bp	cATgAT
$lacI^{ql}$	TTGACA	18 bp	cATgAT
Consensus	**TTGACA**	**17 bp**	**TATAAT**

strand and the active site of the RNA polymerase essential for the following mRNA synthesis (de Haseth *et al*., 1998; Tomsic *et al*., 2001; Tsujikawa *et al*., 2002). The initiation step is characterized

by many rounds of abortive synthesis, but once the RNA polymerase has synthesized a polynucleotide of 13-15 base pairs length, the transcription complex undergoes promoter clearance and the elongation step begins (Carpousis and Gralla 1980; Krummel and Chamberlin 1989; Hsu *et al*., 2003).

Cloning of TGase gene in order to appreciate on structural and functional relationships of TGase gene and the expression level of the recombinant TGase may have potential for industrial-scale production. In general, microbial TGase gene was studied using genomic library construction or amplification using PCR technique.

4.1 Characteristics of expressed TGase

4.1.1 TGase activity

TGase activities from *S. cimmmonemn* CBS 683.68 was 120-fold with a yield of 33% from the culture supernatant (Duran *et al*., 1998). The activity of *stg*A in *S. lividans* TK24 was 1.2 U/ml after culture for 7 day and was 4.6-fold higher than that of the control strain. TGase activities of *stg*A in *S. platensis* YK-2 and *S. cinnamoneus* ATCC 11874 were reached at 2.9 and 2.3 U/ml, after 5 and 3 days cultivation, respectively These results indicated that the activities of TGases were increased by 32 and 22% as compared to those of control strains, and were 2.4 and 1.9-fold higher compared to those of *S. lividans* exconjugants, respectively (Bae *et al*., 2012). TGase activity (2.2 U/ml) of the recombinant appeared after 48 h cultivation. The activity was about 3.3-fold of that produced by *S. platensis* M5218 (maximal activity 0.66 U/ml after 52 h cultivation) (Lin *et al*., 2006). The purified pelB-pro-MTG-His6 from *S. mobaraensis* in *E. coli* was activated by trypsin. The final product was not absolutely pure, showing some degradation of the microbial TGase. However the specific activity of the activated recombinant microbial TGase was determined to be 14 U/mg which is in the range of specific activities published for wild type microbial TGase purified from *S. mobaraensis* (22.6 U/mg (Ando *et al*., 1989)) or the recombinant one from *Corynebacterium glutamicum* (30 U/mg (Date *et al*., 2004)) (Marx *et al*., 2007). The specific activity of the purified TGase from *Streptomyces* S-8112 (15 U/mg protein), the activity level was only 20% of purified enzyme (Kawai *et al*., 1997). TGase activity of *S. netropsis* in culture broth was obtained after 20 to 24 h of cultivation and amounted to approximately 1.32 U/ml of culture broth. The TGase activity was found to decline rapidly in the culture broth and only 15–20% of the maximum activity was detected after 50 h incubation (Yu *et al*., 2008). Enhancement of the TGase activity from *S. fradiae* (3.2 U/ml, 1.3 times that of the original strain) was observed in the recombinant strain, especially during the late stationary phase. Significantly, the specific activity of the TGase in the recombinant strain (3.8 U/mg) is twice that of the original strain (1.9 U/mg) (Liu *et al*., 2006).

4.1.2 Effect of pH and temperature on activity and stability

The optimal pH and temperature of purified TGase from *S. netropsis* for hydroxamate formation were pH 6.0 and 45˚C, respectively. The purified TGase was stable at pH 6.0-9.0 and the activity of the enzyme rapidly declined under acidic condition (pH 3.0-4.0). The activity decreased dramatically when the enzyme was incubated at temperatures higher than 50˚C. The stability of the purified enzyme was enhanced by adding equal volume of glycerol to the enzyme solution. An approximate 10% loss in activity was observed during storage at -20˚C for 8 months (Yu *et al*., 2008).

5. Housekeeping genes

Housekeeping genes are typically constitutive genes that required for the maintenance of basal cellular functions. They are important for the survival of a cell, regardless of its specific role in the tissue or organism. Thus, housekeeping genes are expressed in all cells of an organism under normal and pathophysiological conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal. The candidate reference genes were chosen from various functional classes represent in many bacteria (Galisa *et al*., 2012). The category of housekeeping genes are classified using functions of genes. The summary of some housekeeping gene in bacteria and their function (Gil *et al*., 2004) is shown in the Table 1.5. In addition, 16S ribosomal RNA (rRNA) gene that has been widely used for study of the identify bacterial species and taxonomy (Choi *et al*., 1996; Munson *et al*., 2004; Petti *et al*., 2005; Schmalenberger *et al*., 2001). In general, 16S rRNA genes of bacteria contain nine hypervariable regions, which can be used to consider the sequence diversity among different bacterial species and use for bacterial identification (Peer *et al*. ,1996). Hypervariable regions are flanked by conserved stretches of most bacteria. They can be amplified by PCR approach using universal primers (Baker *et al*., 2003; Lu *et al*., 2000; McCabe *et al*., 1999; Munson *et al*., 2004).

Table 1.5 Summary of some housekeeping genes in bacteria and their function.

Category	Subcategory	Gene	Protein function
DNA metabolism	Basic replication machinery	*gyr*B	DNA gyrase, B subunit
RNA metabolism	Basic transcription machinery	*dea*D	ATP-dependent RNA helicase
		*rpo*A	RNA polymerase, α subunit
		*rpo*B	RNA polymerase, β subunit
		*rpo*C	RNA polymerase, β' subunit
	Translation: aminoacyl-tRNA synthesis	*ile*S	Isoleucyl-tRNA synthase
		*leu*S	Leucyl-tRNA synthase
	Translation factors	*fus*A	Elongation factor G
		*inf*A	Initiation factor IF-1
		*inf*B	Initiation factor IF-2
		*inf*C	Initiation factor IF-3
		*lep*A	GTP binding elongation factor
Protein processing, folding, and secretion	Protein posttranslational modification	*map*	Methionine aminopeptidase
	Protein folding	*grp*E	Hsp70 cochaperone
	Protein translocation and secretion	*fts*Y	Signal recognition particle receptor
		*sec*A	Preprotein translocase subunit (ATPase)
		*sec*E	Membrane-embedded preprotein translocase subunit
		*sec*Y	Membrane-embedded preprotein translocase subunit
Energetic and intermediary metabolism	Proton motive force generation	*atp*D	ATP synthase β chain

6. Export of protein

Many proteins have a signal sequence (leader sequence or leader peptide), which are a short amino acid sequence (usually 16-30 amino acids). Its function is a postal code for the target organelle. The translation of mRNA to protein using a ribosome occurs within the cytosol (Blobel and Sabatini, 1970). The proteins are synthesized in a different organelle and are transported as two ways depend on the type of protein: 1) co-translational translocation (translocation during the process of translation) and 2) post-translational translocation (translocation after the process of translation is complete). The proteins are synthesized from endoplasmic reticulum (ER), golgi, endosomes or membrane-bound used the co-translational translocation pathway for export to cytosol. Most secretory proteins use this pathway for translocation. The co-translational pathway begins with the N-terminal signal peptide of the protein bind a signal recognition particle (SRP) while the protein is still synthesized on the ribosome. The synthesis stops while the ribosome-protein complex is transferred to an SRP receptor on the ER in eukaryotes and the plasma membrane in prokaryotes. The nascent protein is inserted into the translocon, a membrane-bound protein is conduced to channel that comprise the Sec61 translocation complex in eukaryotes, and the homologous SecYEG complex in prokaryotes. Some proteins are translated in the cytosol and after that they are transported into the ER or plasma membrane using a post-translational system. The post-translational translocation of prokaryotes needs the cofactors such as SecA and SecB while Sec63 and Sec63 are used in eukaryotes. In addition, proteins targeted to other destinations, for example chloroplasts, mitochondria, peroxisomes or nucleus also use this pathway.

Secretion in bacteria refers to the transport or translocation of molecules, for example proteins or toxin from across the interior (cytosol or cytoplasm) of a bacterial cell to its exterior. Secretion is a very important mechanism in bacterial because it involves in function and operation in their natural surrounding environment for adaptation and survival. There are two major bacterial protein export pathways as follow: the general secretion (Sec) protein export pathway. In bacteria, the Sec pathway is the most important transport way for export the proteins from organelle to the cytosol (Tsirigotaki *et al*., 2017). The mechanism of the Sec pathway

(Figure 1.4a), it is a co-translational mode (1), Sec substrates possessing highly hydrophobic signal peptides (SP) are bound at the ribosome by the signal recognition particle (SRP). Afterward, the ribosome-nascent chain (RNC)-SRP complex bind to the SRP-receptor FtsY and then the RNC is further transferred to the SecYEG translocation pore such that ribosomal exit site is in close proximity to SecYEG. The energy for translocation in the co-translational export mode is provided by further elongation of the substrate at the ribosome. For the posttranslational mode (2), Sec-dependent precursor proteins are kept in an export-competent state by post-translationally interacting proteins (PIP's) such as SecB, the general chaperones GroELS/DnaK–DnaJ–GrpE or the soluble form of SecA. The signal peptide (SP) is bound with the SecA protein which pushes the protein through the SecYEG protein conducting channel in a stepwise and ATP-dependent manner. In addition, SecDF applies a proton motive force (pmf)-dependent pulling force on the substrate from the trans-side of the cytoplasmic membrane (CM). During or shortly after translocation, the signal peptide is removed by signal peptidase (SPase) and the mature protein is released on the trans-side of the CM. Another, the twin-arginine translocation (Tat) protein export pathway. The mechanism of the Tat pathway (Fig. 1.4b), after folding required cofactor insertion, the preproteins contained a signal peptide with a twin-arginine motif (RR) are bound to a receptor complex, it comprises a TatC and TatB. After that homooligomeric complexes of TatA are recruited to the substrate-loaded receptor complex in a proton motive force (pmf)-dependent manner, next, the translocation of the substrate across the CM. After that substrate translocation, the signal peptide is removed using signal peptidase (SPase) and the mature protein is released on the trans-side of the membrane.

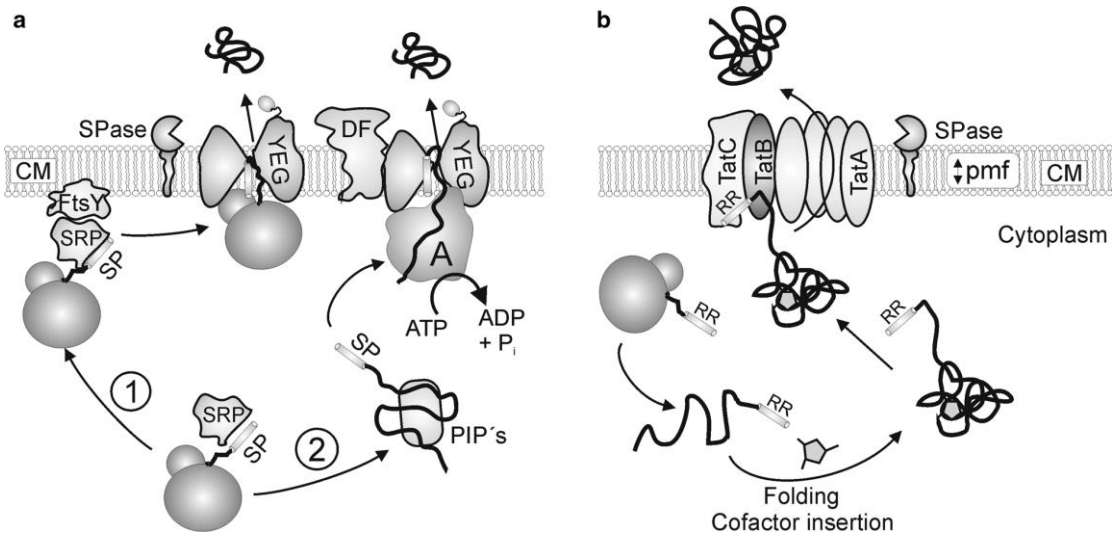


Figure 1.4 Two major bacterial export pathways. (a), the general secretion (Sec) protein export pathway. (b), the twin-arginine translocation (Tat) protein export pathway.

Source: Freudl (2018)

7. Application of microbial TGase

7.1 Meat products

The microbial TGases can be used to produce restructured meat by binding together small pieces of meat. Kuraishi *et al*. (1996) developed a novel meat binding system using microbial TGases and caseinate. Caseinate, when reacted with microbial TGase, becomes viscous, and functions as a glue to bind different foodstuffs together. Using this system, large pieces of restructured meat such as beefsteaks or fish fillets can be produced from fragments. Pieces of meat, including minced meat, can be bound together without any need for sodium chloride or phosphates, yielding healthy meat products. The TGase treatment shows a synergistic effect, when combined with salt and phosphates. The TGase was used to improve functional, textural and gel properties of chicken (Trespalacios and Pla, 2007; Ahhmed *et al*., 2009), beef (Dondero *et al*., 2006; Ahhmed *et al*. 2009), pork and ham (Ávila *et al*., 2010).

7.2 Fishery products

Seki *et al*. (1990) found that endogenous fish TGases caused `suwari' setting and hardening fish protein paste at low temperature through crosslinking. Both endogenous fish TGases and exogenous microbial TGases enable an improvement in the functionality of fish raw materials by increased crosslinking. A combination of microbial TGases injection and tumbling results in reduced loss during thawing and cooking in frozen fish products. Kumazawa *et al*. (1996) determined the ε-(γ-glutamyl)-lysine bonds (G-L bonds) in several fish eggs, and suggested that endogenous TGases may correlate with the texture of raw and processed egg products. It seems that TGase treatment improves and maintains the texture-quality of fish products, which strictly depends on freshness of raw materials. After that, Gonçalves and Passos (2010) studied the influence of three concentrations of commercial TGases in restructured fillet of minced fish from white croacker (*Micropogonias furnieri*). Three concentrations (1.5, 1.0 and 0.5%) of microbial TGases (Active TG-B %v/v and Active TG-BP %w/w) were compared, in order to produce fish restructured product (boneless fillet). Concentration of 1.5% (both enzymes), produced better results. The restructured products were compared by sensory analysis and showed better sensory parameters (appearance, odour, flavor and texture) for samples treated with Active TG-B (solution form).

7.3 Wheat products

Sakamoto (1996) found that treatment of noodles and pasta with microbial TGases prevented the deterioration of texture upon cooking, and improved the strength of the products, even when low-grade flours were used. They also suggested that the loaf volume of several breads might be increased or maintained by the addition of microbial TGases when certain ingredients were substituted or reduced during mixing of the dough. TGgase has the ability to modify wheat protein effectively as a result of the modification of some important physical properties of wheat flour dough, including stickiness, extensibility, and maximum resistance to extention. TGases has proved to be a potential enhancer of baking properties because a very small dosage can cause obvious modification on dough properties (Tseng and Lai, 2002) In 2011, TGases was used to improve dough strength and bread volume (Seravalli *et al*., 2011).

7.4 Antibody

Josten *et al*. (2000) investigated an enzymatic approach to biotinylation using TGases from *S. mobaraense*. This method was exploited for biotinylation using two amino-modified biotin derivatives, biotinamido-5-pentylamin (BIAPA) and biotinoyl-1,8-diamino-3-6-dioxaoctane (BIDADOO) as acyl acceptors and a monoclonal IgG against the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as the acyl donor. Kinetic studies revealed that the TGase-mediated reaction proceeds with low velocity and is almost complete after 34 h. Conjugation ratios ranging from 1.1 to 1.9 biotins per IgG were found by mass spectrometry. To investigate the influence of antibody conjugation on antigen binding a competitive ELISA for the determination of 2,4-D employing microbial TGase-biotinoylated IgGs was developed. In this assay lower limits of detection of 0.3 and 1.0 μg/l of 2,4-D were achieved with BIDADOO- and BIAPA-modified antibodies, respectively.

7.5 Surimi

Surimi is the minced fish flesh, washed to remove most of lipids, blood, enzymes and sarcoplasmic protein and stabilized for frozen storage by cryoprotectants. The myofibrillar protein in the resulting product is high which process gel forming ability (Benjakul *et al*., 2003). Gelation of fish during setting has close relationship to the formation of cross-link between myosin heavy chain induced by endogenous TGases (Kumazawa *et al*., 1995; Seki *et al*., 1990). To improve the gel properties, some additives have been used in surimi and surimi products to alleviate protein degradation caused by the endogenous proteinase. Bovine plasma protein, porcine plasma protein, egg white and potato powders can be used as food grade inhibitors in surimi (Lee *et al*., 2000; Benjakul *et al*., 2001) Additionally, TGase is able to catalyze the formation of ε-(γ-glutamyl) lysine crosslinks and strengthen the gel (Seki *et al*., 1990; Sakamoto *et al*., 1995). TGases from microorganism such as *S. mobaraense* has been used to increase the gel strength by inducing the polymerization of protein (Washizu *et al*., 1994).

7.6 Dairy products

Milk casein is a very good substrate for TGases, which can be converted it into a heat-resistant, firm gel. Yogurt, a milk gel formed by acidic fermentation with lactic starter, has the disadvantage of serum separation upon change

of temperature, or physical impact. The addition of microbial TGases can overcome this problem by improving the water-holding capacity of the gel. The microbial TGase was used to improve properties of yoghurt (Gauche *et al*., 2009) and modify the functionality of whey proteins (Truong *et al*, 2004; Gauche *et al*., 2008). Microbial TGases also make it possible to produce dairy products, such as ice cream (Rossa *et al*., 2011) and cheese, with low fat content or reduced content of non-fat solids.

Objectives

1. To identify new TGases-producing bacteria
2. To clone and express the TGase gene from the selected bacteria into *Escherichia coli*
3. To purify and characterize the expressed TGase

CHAPTER 2

PROVIDENCIA THAILANDENSIS SP. NOV., ISOLATED FROM SEAFOOD PROCESSING WASTEWATER

2.1 Abstract

The bacterial strain C1112^T was isolated from seafood processing wastewater collected from a treatment pond of the seafood factory in Songkhla Province, Thailand. Phylogenetic analysis based on concatenated sequences from the 16S rRNA gene and five housekeeping genes, *fus*A, *lep*A, *leu*S, *gyr*B and *ile*S respectively showed that the strain C1112^T belonged to the genus *Providencia*, and share 91.75% similarity with *P. stuartii* DSM 4539^T. DNA-DNA hybridization between the strain C1112^T and *P. stuartii* KCTC 2568^T was 48.1% relatedness. Moreover, some results from biochemical properties indicated that the strain C1112^T was distinguished from the phylogenetically closest relatives. The major fatty acids of strain C1112^T were $C_{16:0}$, iso-$C_{15:0}$, $C_{14:0}$ and $C_{17:0}$ cyclo and the DNA G+C content was 41 mol%. Base on the genotypic and phenotypic considerations, it should be classified as a novel species of the genus *Providencia* for which the name *Providencia thailandensis* sp. nov. is proposed. The type strain is C1112^T (= KCTC 23281^T =NBRC 106720^T).

2.2 Introduction

The genus *Providencia,* of the class γ–Proteobacteria, order *Enterobacterials,* and family *Enterobacteriaceae,* currently has eight recognized species (*P. burhodogranariea*, *P. heimbachae*, *P. alcalifaciens*, *P. rustigianii*, *P. sneebia*, *P. rettgeri*, *P. vermicola* and *P. stuartii*) (Euzéby, 2018; Juneja and Lazzaro, 2009; Muller *et al*., 1986; Somvanshi *et al*., 2006). Member of the genus have repeatedly been found in association with humans, insects and many other vertebrate and invertebrate animals in both pathogenic and non-pathogenic contexts (Juneja and Lazzaro, 2009; Muller *et al*., 1986; Somvanshi *et al*., 2006; Yoh *et al*., 2005). The description of several of these species was initially based upon on DNA-DNA reassociation experiments, which indicated their previous misclassification in other

genera or affiliation to other species (Brenner *et al*., 1978; Hickman-Brenner *et al*., 1983; Muller *et al*., 1986). The eight species can be separated based on metabolic characteristics, such as acid production from some carbohydrates and several other standard tests (Juneja and Lazzaro, 2009; Owen *et al*., 1987). *Providencia* sp. C1112 was the Gram-negative and rod-shaped bacterium isolated from seafood processing wastewater, which produced TGase enzyme (H-Kittikun *et al*., 2012). This enzyme has been found to induce cross-linking and gelation of food proteins. TGases obtained from microorganisms does not require calcium for activation, which is of great advantage for the food industry. Because several food-proteins are easily precipitated in the presence of Ca^{2+}, then rendering them less sensitive to the enzymatic reaction (Negus 2001). In order to gain more information on the taxonomic position, the sequences based on concatenated sequences from the 16S rRNA gene and five housekeeping genes and the biochemical properties of the strain $C1112^T$ was analyzed. Comparative analysis revealed that the isolate was distinct from the closest phylogenetic relative, *Providencia stuartii*. A strain was further characterized based on the results of a polyphasic taxonomic study, it can be concluded that the strain represent a novel specie of the genus *Providencia*.

2.3 Materials and Methods

2.3.1 Isolation

The strain $C1112^T$ was isolated from seafood processing wastewater and this strain could produce a transglutaminase (Bourneow *et al*., 2012). Briefly, the strain $C1112^T$ was isolated by means of the usual dilution plating technique from seafood processing wastewater, collected from a treatment pond of the seafood factory in Songkhla Province, Thailand. The sample was plated on nutrient agar (NA) plates and incubated at 37˚C for 24 h. A pure culture was obtained by repeated transfers of separate colonies on NA plates. After that, the TGase-producing strains were selected using a colorimetric hydroxamate assay (Folk and Cole, 1966) of their supernatant from the culture medium.

2.3.2 Morphology, physiology and biochemical characteristics

The colony morphology of the strain C1112^T was determined by examining cells grown on NA for 2 days at 37˚C. The cell size and morphology were determined using scanning electron microscopy of cells grown in NB for 2 days at 37 ˚C with shaking at 150 rpm. The gram reaction was determined by the method of Hucker and Conn (1923). Flagella were examined of cells grown in NB for 1 day at 30˚C without shaking by using the staining method described by Forbes (1981). Growth on MacConkey agar and on bile salt medium was tested. Tests for catalase, oxidase, the hydrolysis of casein, gelatin, starch, Tween 80 and tyrosine, the methyl red/Voges-Proskauer (MR-VP) reaction, tryptophan deaminase activity and utilization of citrate were performed. Hydrogen sulphide production, indole production and cell motility were examined using sulphide-indole-motility (SIM) medium (Barrow and Feltham, 1993). Growth in various salt concentrations (0-15% NaCl), at different pH values (3-13) and at various temperatures (4-50˚C) was tested in NB. All tests were carried out by incubating the cultures at 37˚C, except for the investigation into the effect of temperature upon growth. Additional tests were performed by using the API 20E and 50CH strips (bioMérieux) according to the manufacturer's instructions.

2.3.3 Preparation of DNA, 16S rRNA gene sequencing, partial sequences of five housekeeping genes and phylogenetic analysis

DNA was extracted and purified from their whole cells by the phenol method (Saito and Miura, 1963). 16S rRNA gene sequencing was carried out (Shida *et al*., 1996) by using the 27F and 1488R rRNA primers (Ruiz-Garcia, 2005). The five housekeeping genes were amplified using a set of primer as decribed by Juneja and Lazzaro (2009). The PCR products of 16S rRNA gene and five housekeeping genes were purified and sequenced with ABI 3730XLs by using BigDye v3.1 (Applied Biosystems, Foster, California, U.S.A.) according to the manufacturer's instruction. The 16S rRNA gene sequence (978 nt) and five housekeeping genes (*fus*A, 616 nt; *lep*A, 735 nt; *leu*S 412 nt; *gyr*B, 814 nt; *ile*S 917 nt) were deposited in the GenBank databases under the accession numbers KC447298, KC447299, KC447300, KC447301, KC447302 and KC447303, respectively. The six genes were concatenated and aligned along with the selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the program CLUSTAL_X (version 1.81)

(Thompson *et al*., 1997). Gaps and ambiguous bases were eliminated from the calculations and the distance matrices for the aligned sequences were calculated by the two-parameter method (Kimura, 1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with program MEGA (version 5.1) (Tamura *et al*., 2011). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis (Felsenstien, 1985) based on 1000 samplings.

2.3.4 DNA-DNA hybridization and DNA base composition

Levels of DNA-DNA relatedness were determined by the method of Ezaki *et al.* (1989) using photobiotin and microdilution plates. The DNA G+C content was determined by the method of Tamaoka and Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

2.3.5 Chemotaxonomy

The analysis of cellular fatty acids from cells grown in NB for 24 h at 37˚C was determined by fatty acid methyl ester analysis according to the standard protocol of the Microbial Identification System (MIDI) (Sasser, 1990) at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL, Bangkok, Thailand).

2.4 Results and Discussion

2.4.1 Morphology, physiology and biochemical characteristics

The strain C1112^T was Gram-negative, non-spore-forming and rod shaped which motile with peritrichous flagella (Figure 2.1). It was positive for catalase and oxidase, fermented esculin and utilized citrate. The strain C1112^T could be differentiated from the closest phylogenetic relative, *Providencia stuartii* KCTC 2568^T by means of some phenotypic characteristics (Table 2.1). Consistent with the description of other *Providencia* species, the strain C1112^T developed a characteristic smell on agar containing amino acids (Polster and Svobodova, 1964; Somvanshi *et al.*, 2006).

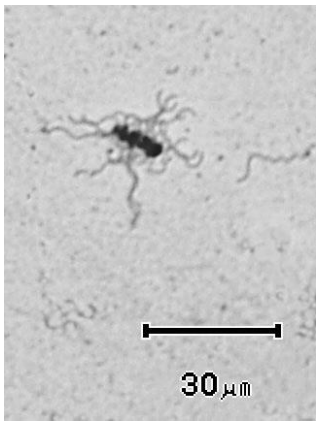


Figure. 2.1 Phase-contrast micrograph of cell of the strain C1112^T grown in NB at 30˚C for 1 day without shaking. Bar: 30 µm.

2.4.2 16S rRNA gene sequencing, partial sequences of five housekeeping genes sequencing and phylogenetic analysis.

The percentage divergence among type strains of described *Providencia* species across the concatenated 16S rRNA gene and five housekeeping genes showed the sequence similarities, strain C1112^T and *Providencia stuartii* DSM 4539^T, *P. rustigianii* DSM 4541^T, *P. rettgeri* DSM 4542^T, *P. alcalifaciens* CIP 8290^T, *P. sneebia* DSM 19967^T, *P. vermicola* DSM 17385^T, *P. burhodogranariea* DSM 19968^T and *P. heimbachae* DSM 3591^T were 91.75, 89.63, 88.54, 88.53, 88.51, 88.45, 88.25 and 87.59%, respectively. The phylogenetic tree base on concatenated 16S rRNA gene and five housekeeping genes (Figure 2.2) was similar to phylogenetic tree given by Juneja and Lazzaro (2009).

Table 2. 1 Differentiation of *Providencia* strains based on biochemical properties.

Characteristic	1	2	3	4	5	6	7	8	9
Citrate utilization	+	+	-	-	-	-	-	-	+
Urea	-	-	-	-	-	+	-	+	-
Indole production	-	+	+	+	+	+	+	+	-
Gelatinase	-	+	-	+	+	+	+	+	+
Acid from:									
D-Lyxose	-	+	-	-	-	-	-	-	-
D-Mannitol	+	-	+	-	+	-	-	+	-
D-Raffinose	+	-	-	-	-	+	-	-	-
D-Xylose	+	-	-	-	-	-	-	+	-
L-Arabinose	+	-	+		-	+	-	-	-
L-Rhamnose	+	-	-	-	-	-	-	-	-
2-Ketogluconate	+	-	+	-	-	-	-	-	-
Arbutin	+	-	-	-	+	-	-	+	+
Cellobiose	+	-	-	-	-	-	-	-	-
Esculin	+	-	-	+	+	+	+	+	+
Glycerol	+	+	-	-	-	+	-	-	-
Mannitol	+	+	-	-	-	-	-	-	-
Salicin	+	-	-	-	+	-	-	+	-
Sorbitol	+	-	-	-	+	+	-	+	-
Sucrose	+	+	-	-	-	-	-	-	-
Mol% DNA G+C	41	40.7[a]	ND	43[a]	ND	40.5[a]	41.8[a]	ND	39.6[a]

1. C1112^T; 2. *Providencia stuartii* KCTC 2568^T; 3. *Providencia vermicola* DSM 17385^T; 4. *Providencia alcalifaciens* JCM 1673^T; 5. *Providencia burhodogranariea* JCM 16940^T; 6. *Providencia rettgeri* JCM 1675^T; 7. *Providencia rustigianii* JCM 3953^T; 8. *Providencia sneebia* JCM 16941^T and 9. *Providencia heimbache* DSM 3591^T; +, positive; -, negative; ND, not determined.

[a]Data from Owen *et al*. (1987)

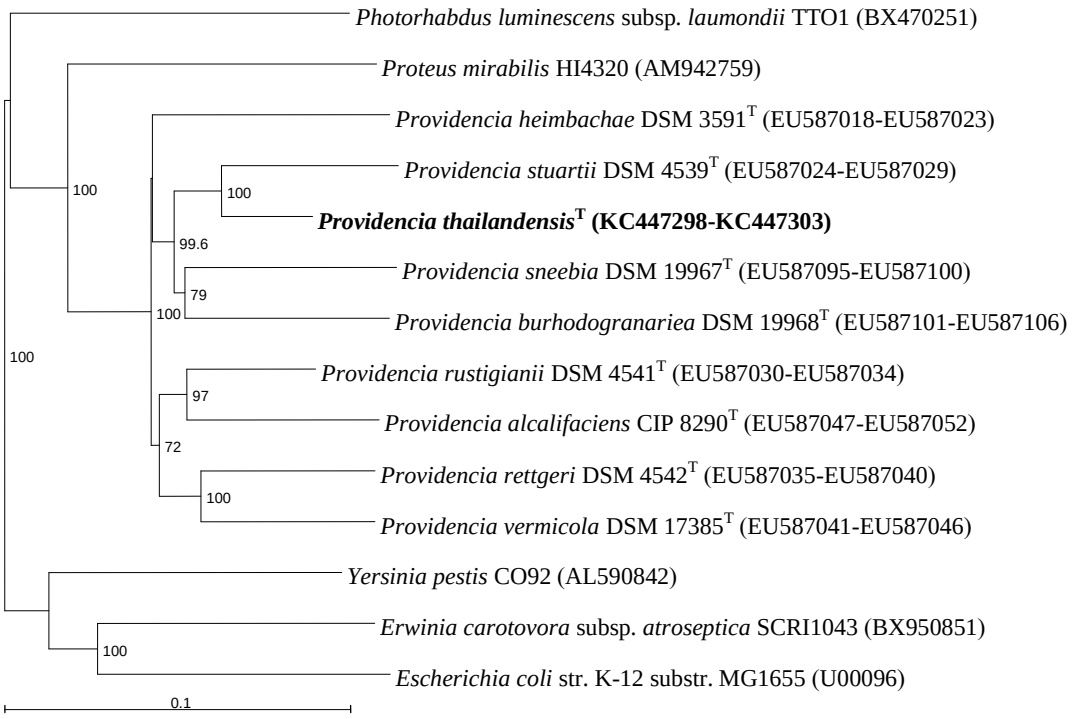


Figure. 2.2 Neighbour-joining phylogenetic tree based on concatenated sequences from the 16S rRNA gene, *fus*A, *lep*A, *leu*S, *gyr*B and *ile*S (4472 nt) of *Providencia thailandensis* sp. nov., recognized *Providencia* species and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. Bar, 0.01 substitution per nucleotide position.

2.4.3 DNA-DNA hybridization and DNA base composition

The DNA-DNA hybridization study revealed that strain C1112^T showed DNA-DNA similarities of 100, 48.1, 42.3, 22.6, 38.7, 39.1, 36.6, 19.1, and 39.7% respectively to the strain C1112^T, *P. stuartii* KCTC 2568^T, *P. vermicola* DSM 17385^T, *P. heimbachae* DSM 3591^T, *P. burhodogranariea* JCM 16940^T, *P. rettgeri* JCM 1675^T, *P. rustigianii* JCM 3953^T, *P. sneebia* JCM 16941^T, and *P. alcaifaciens* JCM 1673^T. A labeled DNA of *P. stuartii* KCTC 2568^T gave DNA-DNA similarities of 46.9 and 100% respectively to strain C1112^T and *P. stuartii* KCTC 2568^T (Table 2.2). This result indicates that the strain C1112^T is significantly distinct from the nearest described relative in *Providencia*. Previous studies have reported reassociation

values that range from 22 to 49% between strains of separate species in this genus (Brenner *et al*., 1978; Hickman-Brenner *et al*., 1983; Juneja and Lazzaro, 2009; Muller *et al*., 1986; Somvanshi *et al*., 2006). In addition, the results from the DNA-DNA hybridizations fall well below the 70% reassociation threshold recommendation of Wayne *et al*. (1987) for designation of a novel species. The G+C content of the stain C1112^T was 41 mol%, a value within the range of (39.6-43.0 mol%) for the genus *Providencia* (Owen *et al*., 1987).

Table 2.2 DNA-DNA relatedness of strain C1112^T and related *Providencia* species.

Strain	DNA-DNA relatedness (%) with labeled strains	
	C1112^T	KCTC 2568^T
C1112^T	100	46.9
P. stuartii KCTC 2568^T	48.1	100
P. vermicola DSM 17385^T	42.3	ND
P. heimbachae DSM 3591^T	22.6	ND
P. burhodogranariea JCM 16940^T	38.7	ND
P. rettgeri JCM 1675^T	39.1	ND
P. rustigianii JCM 3953^T	36.6	ND
P. sneebia JCM 16941^T	19.1	ND
P. alcaifaciens JCM 1673^T	39.7	ND

ND, Not determined.

2.4.4 Chemotaxonomy

The strain C1112^T exhibited a fatty acid pattern characterized by high level of $C_{16:0}$ (27.26%), iso-C_{15} (14.62%), $C_{14:0}$ (13.14%), and $C_{17:0}$ cyclo (11.97%), respectively. Additional fatty acids included $C_{12:0}$ (0.89%), iso-$C_{13:0}$ (2.81%), iso-$C_{14:0}$ (1.62%), iso-$C_{16:0}$ (2.78%), iso-$C_{17:0}$ (3.05%), anteiso-$C_{15:0}$ (1.88%), anteiso-$C_{17:0}$ (0.88%), iso- $C_{17:1}\omega5c$ (0.53%) and $C_{19:0}$cyclo$\omega8c$ (2.99%) were also detected. On the basis of the above phylogenetic results, physiological and biochemical data, cellular fatty acid composition and G+C content, the strain C1112^T should be placed in the genus *Providencia* as a novel species, for which the name *Providencia thailandensis* sp. nov. is proposed.

2.4.5 Description of *Providencia thailandensis* sp. nov.

Providencia thailandensis (thai.lan.den'sis. N.L. masc. adj. *thailandensis* pertaining to Thailand, where the type strain was isolated). Colonies are creamy white, moist, opaque, flatted and undulate margin with 12-19 mm in diameter after 48 h incubation at 37˚C on nutrient agar. Cells are Gram-negative, non-spore-forming, rod-shaped and motile with peritrichous flagella as showed in Figure 2.1. Cells are 0.58-0.60 by 1.40-1.42 μm and occurring single or in pairs. The bacterium does not grow on MacConkey and bile salt agar. The strain is oxidase and catalase positive. The strain gives positive in methyl-red reaction, Voges-Proskauer, hydrolysis of starch, gelatin and casein, ONPG, citrate utilization, arginine dehydrolase and ornithine decarboxylation. The strain is negative for hydrolysis of tyrosine and Tween 80, indole, the production of H_2S, urease and lysine decarboxylation. Growth occurs at 15˚C and 45˚C; optimal growth temperature is 30-37˚C. Growth occurs between pH 4.0-12.5 and optimal pH is 6.0-7.0. Growth occurs in the presence of 0-5% (w/v) NaCl and optimally with 0% NaCl. Tryptophan deaminase is produced. Acid is produced from glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, Methy-α D-glucopyranoside, N-acetylglucosamine, arbutin, esculin, salicin, sucrose, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, amidon (starch), glycogen, gentiobiose and potassium-2-ketogluconate. Acid is not produced from amygdalin, dulcitol, erythritol, L-xylose, D-adonitol, D-arabinose, methyl-βD-xylopyranoside, methyl-αD-mannopyranoside, L-sorbose, inulin, D-melezitose, xylitol, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, D-turanose, potassium-5-ketogluconate and D-lyxose. The G+C content of DNA of strain C1112^T is 41 mol%. The predominant fatty acids are $C_{16:0}$, iso-C_{15}, $C_{14:0}$ and $C_{17:0}$ cyclo. The type strain is C1112^T (KCTC 23281^T, NBRC 106720^T).

2.5 Conclusion

Providencia thailandensis sp. nov. is proposed. The type strain is C1112^T (= KCTC 23281^T =NBRC 106720^T). They are creamy white, moist, opaque, flatted and undulate margin with 12-19 mm in diameter after 48 h incubation at

37˚C on nutrient agar. Cells are Gram-negative, non-spore-forming, rod-shaped and motile with peritrichous flagell. Growth occurs at 15˚C and 45˚C; optimal growth temperature is 30-37˚C. Growth occurs between pH 4.0-12.5 and optimal pH is 6.0-7.0. Growth occurs in the presence of 0-5% (w/v) NaCl and optimally with 0% NaCl.

CHAPTER 3

ENTEROBACTER SIAMENSIS SP. NOV., A TRANSGLUTAMINASE-PRODUCING BACTERIUM ISOLATED FROM SEAFOOD PROCESSING WASTEWATER IN THAILAND

3.1 Abstract

A novel strain of *Enterobacter*, C2361^T, a Gram-negative, non-spore-forming rod-shaped and facultative anaerobic bacterium with capability to produce the transglutaminase (TGase), was isolated from seafood processing wastewater collected from a treatment pond of seafood factory in Songkhla Province, Thailand. Phylogenetic analyses and phenotypic characteristics, including chemotaxonomic characteristics, showed that the strain was a member of the genus *Enterobacter*. The 16S rRNA gene sequence similarities between the strain C2361^T and *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^T and *Enterobacter cloacae* subsp. *dissolvens* LMG 2683^T were 97.5 and 97.5%, respectively. The strain C2361^T showed a low DNA-DNA relatedness with the above-mentioned species. The major fatty acids were $C_{16:0}$, $C_{17:0}$cyclo and $C_{14:0}$. The DNA G+C content was 53.0 mol%. On the basis of the polyphasic evidences gathered in this study, it should be classified as a novel species of the genus *Enterobacter* for which the name *Enterobacter siamensis* sp. nov. is proposed. The type strain is C2361^T (= KCTC 23282^T = NBRC 107138^T).

3.2 Introduction

The genus *Enterobacter* was established by Hormaeche and Edwards (1960). It belongs to the family *Enterobacteriaceae* of the class γ–Proteobacteria and accommodates a number of species of heterotrophic, Gram-negative, non-spore forming, rod-shaped and facultative-anaerobic bacteria. Member of the genus *Enterobacter* are free-living as well as that are capable of causing diseases in plant and animals (Brenner *et al*., 1980; Brenner *et al*., 1986; Ewing and Fife, 1968; Hoffman *et al*., 2005; Madhaiyan *et al*., 2010; Manter *et al*., 2011; Stephan *et al*., 2008).

In our efforts to screen for efficient TGase-producing bacteria from seafood processing wastewater collected from a treatment pond of seafood factory in Songkhla Province, Thailand, one isolate designated as C2361^T was obtained. Biochemical analyses revealed this strain to be affiliated with the genus *Enterobacter*. The strain was examined using phenotypic, physiological, and chemotaxonomic test and by phylogenetic analysis. We report that stain C2361^T should be classified in the genus *Enterobacter* as a novel species, *Enterobacter siamensis* sp. nov.

3.3 Materials and Methods

3.3.1 Isolation of bacterial strain and culture conditions

Strain C2361^T, a TGase-producing bacterium was isolated from seafood processing wastewater collected from a treatment pond of seafood factory in Songkhla Province, Thailand using a standard dilution plating method on nutrient agar (NA) and incubated at 37˚C for 24 h. The capability to produce the TGase of C2361^T was tested by the hydroxamate assay as described by Folk and Cole (1965). The strain was routinely cultured on NA plate at 37˚C and maintained in nutrient broth supplemented with 20% glycerol (v/v) at -70˚C. This isolate was deposited in the Korean Collection for Type Cultures and Biological Resource Center (KCTC 23282^T = NBRC 107138^T).

3.3.2 Morphological, physiological and biochemical characteristics

Cell morphology was observed using scanning electron microscopy. The gram reaction was determined by the method of Hucker and Conn (1923). Flagella were examined by using the staining method described by Forbes (1981). The morphology of C2361^T colonies was determined by examining cells grown on NA for 2 days at 37˚C. Growth on MacConkey agar and bile salt medium was tested. Anaerobic growth was tested by incubating cultures on NA plates in an anaerobic jar with Anaerocult (Merck). Tests for catalase, oxidase, the hydrolysis of casein, gelatin, starch, Tween 80 and tyrosine, the methyl red/Voges-Proskauer (MR-VP) reaction, tryptophan deaminase activity and utilization of citrate were performed as described by Barrow and Feltham (1993). Hydrogen sulphide production, indole production and cell motility were examined using sulphide-indole-motility (SIM) medium (Barrow and Feltham, 1993). Growth in various salt concentrations (0-15% NaCl), at different

pH values (4-11) and at various temperatures (4-50˚C) was tested in NB. All tests were carried out by incubating the cultures at 37˚C, except for investigations into the effect of temperature upon growth. Moreover, API 20E and 50CH strips (bioMérieux) were used to determine the biochemical characteristics according to the manufacturer's instructions.

3.3.3 Chemotaxonomic characteristics

Cellular fatty acid composition of cells grown in NB for 24 h at 30 ˚C was determined by fatty acid methyl ester analysis according to the standard protocol of the Microbial Identification System, MIDI version 6.1, Method RTSBA6. (Sasser, 1990) at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL, Bangkok, Thailand). Fatty acid content was compared to the fatty acid database in the Microbial Identification System, Sherlock version 6.1 (MIDI).

3.3.4 Preparation of DNA and DNA base composition

DNA of strain C2361^T was extracted from 12-16 h cells grown in NB and purified by the phenol method (Saito and Miura, 1963). The base composition of DNA was determined by the method of Tamaoka and Komagata (1984), with the modification that DNA was hydrolyzed and the resulting nucleotides were analyzed by reversed-phase HPLC.

3.3.5 16S rRNA gene sequencing and phylogenetic analysis

The phylogenetic position of the isolated was studied by the standard analysis based on the 16S rRNA gene sequence. 16S rRNA gene sequencing was carried out by using the 27F and 1488R rRNA primers (Ruiz-Garcia *et al*., 2005). The PCR product was purified and sequenced with ABI 3730XL by using BigDye v3.1 (Applied Biosystems, Foster, California, U.S.A.) according to the manufacturer's instruction, by the following primers: 27 F, 421F and 1488R rRNA primers (Ruiz-Garcia *et al*., 2005). The 16S rRNA gene sequence (1429 bases) was deposited in the DDBJ databases under the accession numbers HQ888848. The sequences obtained were aligned to reference 16S rRNA gene sequences available in the GenBank/EMBL/DDBJ databases by using the program CLUSTAL_X (version 1.81) (Thompson *et al*., 1997). Gaps and ambiguous bases were eliminated from the calculations and a neighbour-joining phylogenetic tree was constructed by the method (Saitou and Nei, 1987) using the program MEGA 4 (Tamura *et al*., 2007). The

confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis (Felsenstien, 1985) based on 1000 samplings. The DNA-DNA similarity was calculated by EzTaxon (Server 2.1) (Chun *et al*., 2007).

3.3.6 DNA-DNA hybridization

DNA hybridization was carried out by photobiotin-labelling method with microdilution plates (Ezaki *et al*., 1989).

3.4 Results and Discussion

3.4.1 Morphological, physiological and biochemical characteristics

A TGase-producing bacterium, strain C2361^T was Gram-negative rod and facultative anaerobic. Cell size is 0.5-0.6 by 1.2-1.6 μm and occurring single or in pairs (Figure 3.1A). The cell was motile with peritrichous flagella (Figure 3.1B). They fermented esculin, utilized citrate and grew well on MacConkey agar. The strain C2361^T could be differentiated from other phylogenetically related species of the genus *Enterobacter* by means of some phenotypic characteristics as shown in Table 3.1.

3.4.2 Chemotaxonomic characteristics

The strain C2361^T exhibited a fatty acid pattern characterized by high levels of $C_{16:0}$ (31.02%), $C_{17:0}$cyclo (19.55%) and $C_{14:0}$ (12.08%). Additional fatty acid included $C_{19:0}$cycloω8c (2.01%), $C_{12:0}$ (1.75%), $C_{17:0}$ (0.93%), $C_{18:0}$ (0.46%), $C_{13:0}$ (0.45%), $C_{18:1}$ω9c (0.38%), $C_{16:1}$ω5c (0.18%), $C_{17:1}$ω8c (0.15%), $C_{15:1}$ω8c (0.11%), $C_{19:0}$ (0.07%), iso-$C_{19:0}$ (0.06%), iso-$C_{15:0}$ (0.05%), iso-$C_{16:0}$ (0.05%), iso-$C_{17:0}$ (0.05%) and $C_{20:1}$ω7c (0.03%) were also detected. This is a typical fatty acid pattern of a member of the genus *Enterobacter* and related genera. Comparison of cellular fatty acid composition between strain C2361^T and related *Enterobacter* species (*E. cloacae* subsp. *dissolvens* and *E. cloacae* subsp. *cloacae*) are shown in Table 3.2.

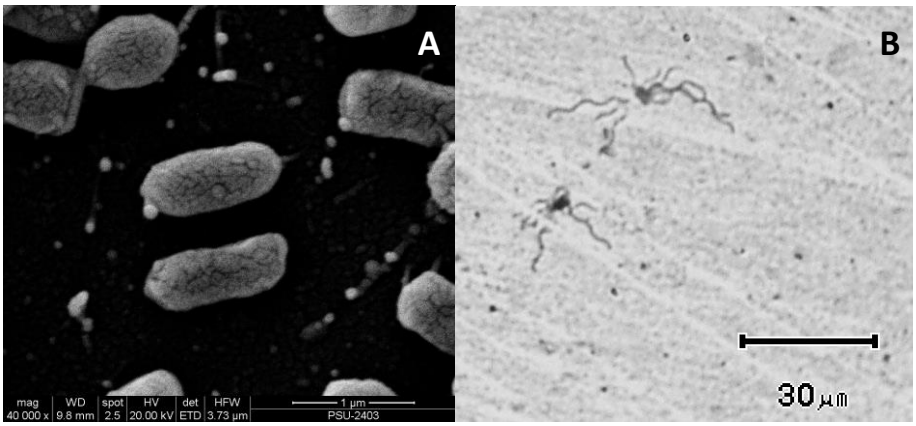


Figure 3.1 Morphology of *E. siamensis* C2361^T. (A) Scanning electron micrograph of cells grown in NB for 1 day at 30˚C with shaking at 150 rpm and (B) phase-contrast micrograph of cells grown in NB for 1 day at 30˚C without shaking.

3.4.3 DNA base composition

The DNA G+C mol% of the strain C2361^T was 53%, which was similar to the range reported for *E. cloacae* subsp. *cloacae* (53%) and *E. cloacae* subsp. *dissolvens* (55%) (Hoffmann *et al*., 2005). These values were also consistent with the DNA G+C mol% of the other members of genus *Enterobacter* that fall within a range of 52-60 mol% (Hoffmann *et al*., 2005; Kampfer *et al*., 2005; Stephan *et al*., 2007; Stephan *et al*., 2008). Generally, bacteria of the same genus will have G+C mol% that are within 10% of each other, while bacteria that are within the same species may have G+C mol% values that differ by up to 5% points (Tamaoka and Komagata, 1984).

Table 3.1 Differential characteristics of strain C2361^T and related *Enterobacter* species.

Characteristics	1	2	3
Esculin hydrolysis	+	-	+
ONPG	+	-	+
Carbon source utilization			
L-Arabinose	+	+	-
Arbutin	+	-	+
D-Fructose	-	+	-
Gentiobiose	+	-	-
D-Glucose	+	+	-
D-Lyxose	+	-	-
D-Mannitol	+	+	-
D-Mannose	+	+	-
L-Rhamnose	+	+	-
D-Ribose	+	+	-
D-Turanose	+	-	-
DNA G+C content (mol%)	53	55	53

Strains: 1, *Enterobacter siamensis* C2361^T; 2, *E. cloacae* subsp. *dissolvens* JCM 6049^T; 3, *E. cloacae* subsp. *cloacae* KCTC 2361^T. +, Positive reaction; -, negative reaction.

Table 3.2 Cellular fatty acid composition of strain C2361^T and related *Enterobacter* species.

Fatty acids	1	2	3
Saturated straight-chain			
$C_{12:0}$	1.75	4.1	3.02
$C_{14:0}$	12.08	6.8	9.97
$C_{16:0}$	31.02	-	31.61
Unsaturated straight-chain			
$C_{19:0}$ cyclo ω8c	2.01	-	2.56
Saturated branched-chain			
$C_{17:0}$ cyclo	19.55	5.2	22.33
Hydroxylated fatty acids			
$C_{14:0}$ 2-OH	1.2	-	-
Summed feature 3[a]	8.09	24.6	5.37
Summed feature 8[b]	10.09	-	15.29

Strains: 1, *Enterobacter siamensis* C2361^T; 2, *E. cloacae* subsp. *dissolvens* JCM 6049^T (Madhaiyan *et al*., 2010); 3, *E. cloacae* subsp. *cloacae* KCTC 2361^T. Value are percentages of total fatty acids; -, not detected; fatty acids amounting to < 0.01% in all strains tested are not listed.

[a]Summed feature 3 contains $C_{16:1}\omega7c/C_{16:1}$; [b]Summed featured 8 contains $C_{18:1}\omega7c$.

3.4.4 Phylogenetic analysis

The 16S rRNA gene sequences of the strain C2361^T was grouped most closely with a cluster containing *E. cloacae* subsp. *cloacae* ATCC 13047^T and *E. cloacae* subsp. *dissolvens* LMG 2683^T with 97.5 and 97.5% similarity, respectively (Figure 3.2). 16S rRNA gene sequence analysis indicates that the strain belongs to the genus *Enterobacter*. Based on this result alone, it cannot unequivocally be allocated to a finer taxonomic level. To confirm finally that this strain represented an independent genospecies within the genus *Enterobacter*, DNA-DNA hybridization with two representative strains was performed.

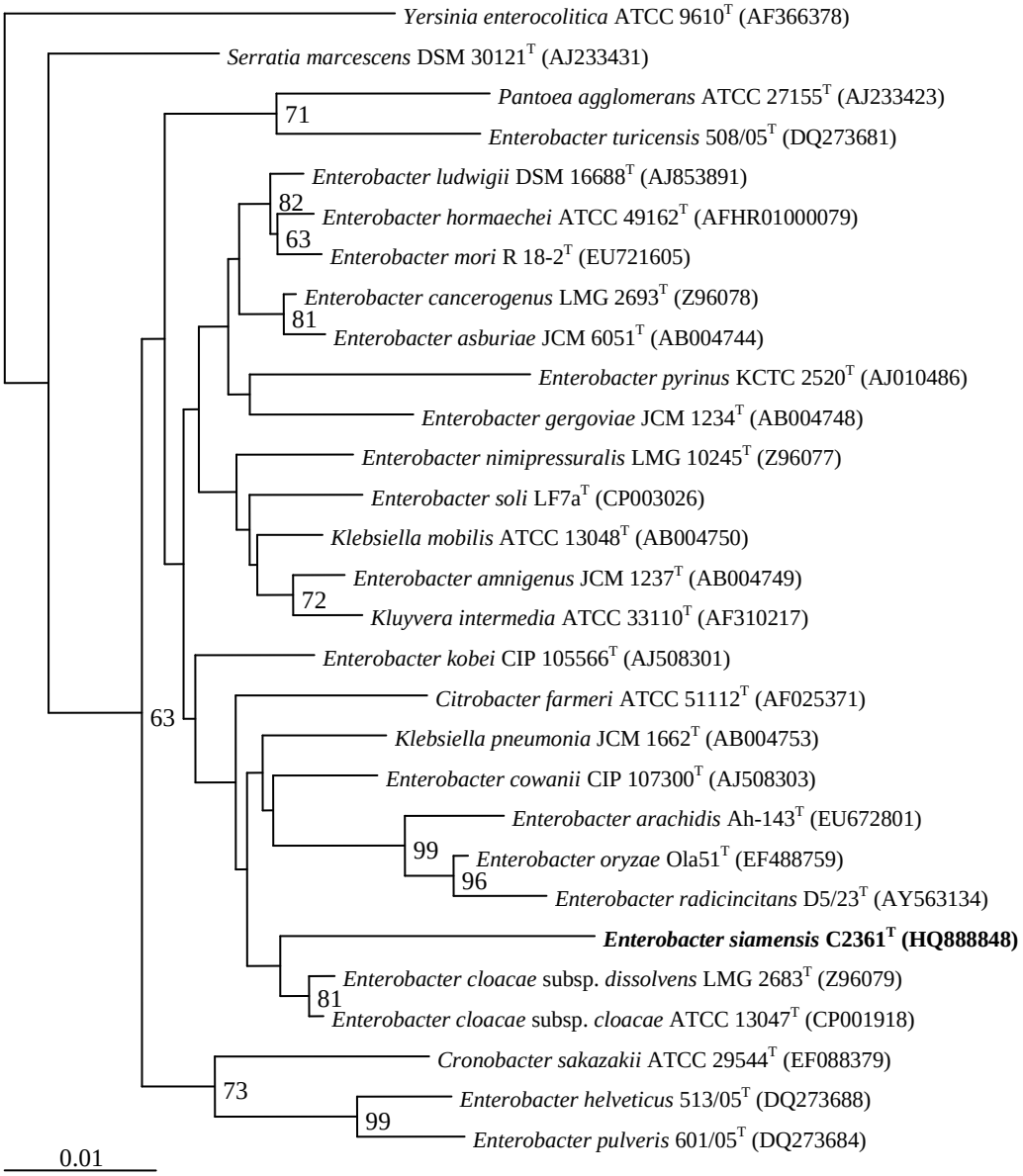


Figure 3.2 A Neighbour-joining tree showing the phylogenetic relationships of *Enterobacter siamensis* sp. nov., recognized *Enterobacter* species and related taxa. Bootstap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. *Yersinia enterocolitica* ATCC 9610^T (AF366378) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

3.4.5 DNA-DNA hybridization

The DNA-DNA hybridization results revealed that the strain C2361^T and *Enterobacter cloacae* subsp. *cloacae* showed a DNA-DNA relatedness of 46.6 ± 0.00 % while strain C2361^T and *Enterobacter cloacae* subsp. *dissolvens* show 48.8 ± 0.02 % DNA-DNA relatedness (Table 3.3). The DNA-DNA relatedness between the strains of the different genospecies falls within a range of 46.6 - 48.8 % which is clearly below 70%, the generally accepted limit for species delineation (Wayne *et al*., 1987).

Based on the results of polyphasic analysis, strain C2361^T should be placed in the genus *Enterobacter* as a novel species, for which the name *Enterobacter siamensis* sp. nov has been proposed.

Table 3.3 DNA-DNA relatedness of strain C2361^T and related *Enterobacter* species.

Strain	DNA-DNA relatedness (%) with labeled strains[a]	
	C2361^T	KCTC 2361^T
C2361^T	100 ± 0.02	47.5 ± 0.15
E. cloacae subsp. *cloacae* KCTC 2361^T	46.6 ± 0.00	100 ± 0.04
E. cloacae subsp. *dissolvens* JCM 6049^T	48.8 ± 0.02	68.8 ± 0.22

[a]Values are expressed as the means of three determinations.

3.4.6 Description of *Enterobacter siamensis* sp. nov.

Enterobacter siamensis (si.am.en'sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, where the type strain was isolated). Cells are Gram-negative, facultative anaerobic, rod-shaped and motile with peritrichous flagella. Colonies on nutrient agar are creamy white, mucoid, translucent, raised, entire margin, and 5-9 mm in diameter after 48 h incubation at 37˚C. Anaerobic growth is occurred. The bacterium grows on MacConkey agar but not on bile salt medium. The strain tested produced catalase and oxidase. Tests for hydrolysis of esculin, Voges-Proskauer, ONPG, citrate utilization, arginine dihydrolase and ornithine decarboxylation are positive while negative for hydrolysis of tyrosine, gelatin, casein, starch and Tween 80, methyl-red reaction, indole, the production of

H_2S, urease and decarboxylation of lysine. Growth occurs at 10-45˚C; optimal growth temperature is 37˚C. Growth occurs between pH 4.0-10.5 and optimal pH is 6.0-7.0. Growth occurs in the presence of 0-7.5% (w/v) NaCl and optimally with 0% NaCl. Tryptophan deaminase is produced. Acid is produced from amygdalin, arbutin, D-cellobiose, D-galactose, D-glucose, D-lactose, D-lyxose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-ribose, D-saccharose, D-sorbitol, D-trehalose, D-turanose, D-xylose, esculin, gentiobiose, glycerol, inositol, L-arabinose, L-rhamnose, Methy-α D-glucopyranoside and salicin. Acid is not produced from amidon (starch), D-adonitol, D-arabinose, D-arabitol, D-fructose, D-fucose, D-melezitose, D-tagatose, dulcitol, erythritol, glycogen, inulin, L-arabitol, L-fucose, L-sorbose, L-xylose, methyl-αD-mannopyranoside, methyl-βD-xylopyranoside, N-acetylglucosamine, potassium-2-ketogluconate, potassium-5-ketogluconate, potassium gluconate and xylitol. The DNA G+C content is 53.0 mol%. Major cellular fatty acids are $C_{16:0}$, $C_{17:0}$cyclo and $C_{14:0}$. The type strain is C2361^T (KCTC 23282^T, NBRC 107138^T).

3.5 Conclusion

Enterobacter siamensis are Gram-negative, facultative anaerobic, rod-shaped and motile with peritrichous flagella. Colonies on nutrient agar are creamy white, mucoid, translucent, raised, entire margin, and 5-9 mm in diameter after 48 h incubation at 37˚C. The DNA G+C content is 53.0 mol%. Major cellular fatty acids are $C_{16:0}$, $C_{17:0}$cyclo and $C_{14:0}$. The type strain is C2361^T (KCTC 23282^T, NBRC 107138^T).

CHAPTER 4

GENE CLONING AND EXPRESSION OF TRANSGLUTAMINASE GENE FROM *PROVIDENCIA THAILANDENSIS* AND *ENTEROBACTER SIAMENSIS*

4.1 Abstract

Transglutaminase (TGase, EC 2.3.2.13) is a robust enzyme applied in foods, pharmaceuticals, textiles, and biomedicine. It catalyzes the formation of an isopeptide bond between glutamine and lysine residues. It is used In this study, TGase genes of *Providencia thailandensis* and *Enterobacter siamensis* were amplified by specific and degenerate primers using 2×Taq Master Mix (Vivantis) and Takara *Ex Taq* as DNA polymerase in PCR reactions. 1,100-bp and 1,200-bp amplified products from *E. siamensis* genomic DNA, using F1R1 and F2R1 primer with 2×Taq Master Mix, showed 96 and 71% sequence similarity to ABC transporter permease *of E. cloacae* and hypothetical protein of *Enterobacteriaceae*, respectively. In addition, 1100-bp amplified product from *P. thailandensis* genomic DNA, using tgFtgR2 primer with Takara *Ex Taq* DNA polymerase, was ligated with pSSBm97 and expressed into *B. megaterium* YYBm1. However, TGase activity could not detect from the expressed protein. Due to *P. thailandensis* and *E. siamensis* are novel species, therefore no information regarding their TGase genes could be acquired from the database. This makes it quite difficult to design primers for TGase gene amplification from these two bacteria.

4.2 Introduction

TGases are a family of enzymes (EC 2.3.2.13) that catalyze an acyl-transfer reaction between the γ-carboxamide group of a protein- or peptide-bound glutamine and the ε-amino group of a lysine residue (Rachel and Pelletier, 2013). TGases have been identified in different sources, including microorganisms, plants, invertebrates, and mammals (Shleikin and Danilov, 2011). Nowaday, TGases is found as two forms of the enzyme: first is a calcium-dependant TGase found in tissues of animals and humans. The later is a calcium-independent, microbial TGase, which was

first isolated from *Streptomyces mobaraense* (Ando *et al.*, 1989) and has since been isolated from other strains, including *S. hygroscopicus* (Cui *et al*., 2007) and *Bacillus subtilis* (Zhang et *al*., 2009). Both types of TGase have been studied extensively in industry. *Streptomyces* sp. TGase has been widely used in the food industry for improving of the functional properties of food products (Yokoyama *et al*., 2004), and it was used in textiles and leather processing (Zhu and Tramper, 2008). The microbiological media used to culture *Streptomyces* strains are not attractive from an economical point of view because the large amount of costly nutrients that are required, such as peptone and yeast extract. The cost of a culture medium may comprise almost 30 % of the total costs of the whole biosynthesis process (Téllez-Luis *et al*., 2004). Therefore, it is pleasing to develop an efficient and easy to use expression system for the TGase production. Processes of the expression and purification of genes encoding TGase biosynthesis have been carried out with *S. lividans* (Washizu *et al*,. 1994; Lin *et al*. 2004), *E. coli* (Liu *et al.*, 2011a; Yokoyama *et al*., 2000) and *Corynebacterium glutamicum* (Date *et al*., 2004). Recently, this enzyme is produced from *S. mobaraense*. It would be useful and practical to develop a more effective system of TGase production. TGase is naturally synthesized as a pro-TGase that is processed by removal of the N-terminal pro-peptide (Marx *et al*., 2007). Several studies have shown that the pro-peptide is essential for overexpression of TGase in *E. coli* (Yu *et al*., 2008; Marx *et al*., 2007; Yokoyama *et al*., 2000). This is a new technique of co-expression involving the direct production of active TGase. In this study, TGase gene from *P. thailandensis* and *E. siamensis* were cloned and expressed.

4.3 Materials and Methods

4.3.1 Enzyme assay

The pro-TGase was activated according to slightly modified method of Marx *et al*. (2008). The pro-TGase was activated using the trypsin at a final concentration of 0.5 µg/µl and incubated at 37˚C for 30 min. TGase activity was assayed by hydroxamate method (Folk and Cole, 1966). Briefly, 50 µl of TGase was mixed with 12 µl of the mixture (174 mM Tris buffer (pH 6.0), 87 mM hydroxylamine, 8.7 mM glutathione, 31 mM CBZ-Gln-Gly and 4 mM calcium

chloride). Then reaction mixture was incubated at 37˚C for 10 min. Thereafter, 94 µl of 12% TCA solution and 94 µl of $FeCl_3$ in 100 mM HCl was added to the reaction mixture, respectively. After 5 min, the mixture was centrifuged at 10,000×*g* for 5 min and measured the absorbance at 525 nm. One unit of TGase activity was defined as the amount of the enzyme used to produce 1 µmol of hydroxamic acid per min at the assay conditions.

4.3.2 Cloning of TGase gene using PCR amplification

4.3.2.1 Primer designing

4.3.2.1.1 Specific primers

The TGase gene of *Streptomyces* sp., *Saccharomonospora* sp. and *Bacillus* sp. were searched from GenBank. The primers were then designed using CLUSTAL_X (version 1.81) program and PerlPrimer v1.1.10.

4.3.2.1.2 Degenerate primers

The TGase gene of *Streptomyces* sp., obtained from GenBank, was converted to protein sequence using ExPazy program and aligned by T-coffee program. The primers were designed using degenerate primer as conserve region of TGase gene.

4.3.2.2 DNA extraction

Genomic DNA was prepared by the phenol method of Saito and Miura (1963) with some modifications. Cells were harvested after 12 h of cultivation and suspension in 2 ml of saline-EDTA buffer pH 8.0. Then 100 µl of 10% SDS was added and incubated at 80˚C for 5 min. The phenol extraction was carried out by adding an equal volume of phenol: chloroform (1:1) to the sample. The upper layer of the mixture was collected after centrifugation at 12,000 rpm for 5 min. Genomic DNA was precipitated with two volume of ice cold absolute ethanol and dried at room temperature. Genomic DNA was dissolved in 0.1× saline-sodium citrate (SSC) buffer. After dissolution, the genomic DNA solution was treated with RNase A and protease K solution and incubated at 37˚C for 1 h. The phenol extraction and ethanol precipitation was performed and genomic DNA was stored in 1×TE buffer at -20˚C.

4.3.2.3 Amplification of TGase gene by PCR technique

The genomic DNA of *P. thailandensis* and *E. siamensis,* used as DNA template, were amplified by using design primers shown in Table 4.1 and 4.2. In this

study, two DNA polymerase were used, namely 2×Taq Master Mix (Vivantis Technologies Sdn. Bhd. Malaysia) and Takara *Ex Taq* DNA polymerase, with the buffer system (Takara Mirus Bio Corporation, Madison, WI). Amplification reaction was carried out in 25 µl volume, the final PCR mixture comprised of 1× *Taq* buffer (with 1.5 mM $MgCl_2$), 200 µM concentration of each deoxynucleoside triphosphate, 0.2 µM concentration of each primer, 1 unit of *Taq* DNA polymerase and 50 ng of DNA template. Amplification was carried out in a thermocycler with different condition, depend on the primers. Then PCR products were analyzed by agarose gel electrophoresis using 2% agarose gel.

4.3.2.4 Competent cells preparation

The *E. coli* strain 10G was grown in Super Optimal Broth (SOB) with shaking at 250 rpm, at 37˚C, for 16 h. Then the suspension was transferred to SOB at a ratio of 1:250 of suspension:SOB and shaked at 250 rpm, at 37˚C until the OD_{600} of the culture was reached to 0.2-0.4. The culture was incubated on ice for 15 min and centrifuged at 3,500 rpm, 4˚C for 10 min. The medium was removed from the cell pellets. The pellets was resuspended in 100 ml of RF1 and incubated on ice for 15 min. The suspension was also centrifuged at 3,500 rpm, 4˚C for 10 min. The supernatant was removed from the pellets. The pellets were dissolved in 4 ml of RF2. The suspension was aliquot to eppendorf and stored at -80˚C (Robertson *et al*., 1997).

4.3.2.5 Plasmid digestion with restriction enzymes

The plasmids were cut with restriction enzymes. The plasmid digestion reaction consisted of 1× buffer, 1 unit of restriction enzymes and 5 µg of plasmid DNA in the total volume of 15 µl. The reaction was incubated at 37˚C overnight. The restriction enzyme was heat to inactivate at 80˚C for 20 min. Then sizes of digested plasmids were compared with DNA markers on agarose gel (Robertson *et al*., 1997).

4.3.2.6 Ligation of DNA

The ligation reaction consisted of 1× ligation buffer, digested DNA fragment and plasmid vector at 3:1 ratio and 1 unit of T4 DNA ligase in the total volume of 10 µl. The reaction was incubated at 4˚C overnight (Robertson *et al*., 1997).

4.3.2.7 Transformation

The competent cells were taken from -80˚C and kept on ice. The plasmid DNA was added to the competent cells and incubated on ice for 30 min. Then the mixtures were incubated at 42˚C for 90 sec and further incubated on ice for 5 min. After that 800 µl of Super Optimal broth with Catabolite repression (SOC) medium was added to the mixture and incubated at 37˚C with shaking at 250 rpm for 1 h. The cell was harvested by centrifugation at 4,000 rpm for 5 min. The broth was removed from the cell pellets. The cell pellets were resuspened in 200 µl of fresh broth. The 100 µl of the suspension was spreaded on LB (Luria-Bertani) plates with appropriate antibiotic added. The plates were then incubated at 37˚C for 12-16 h. (Robertson *et al*., 1997).

4.3.2.8 Plasmid extraction

The plasmids were extracted by Plasmid Miniprep Plus Purification Kit (GMbiolab, Co., Ltd., Taiwan) according to the manufacturer's instruction. Plasmids size was analysis by agarose gel electrophoresis.

4.3.3 Expression of pro-TGase

In order to expressed TGase, the recombinant DNAs were transformed into *B. megaterium* YYBm1. The expression strain was culture in a 5 ml LB medium containing 12 µg/ml ampicillin at 37˚C, 225 rpm. After 12 h of incubation, the culture was transfer into a 250 ml flask containing 50 ml LB medium and 12 µg/ml tetracycline at 37˚C, 225 rpm. When the OD_{600} reached 0.4, 0.5% xylose (w/v) was added and the culture was incubated at 37˚C, 225 rpm for 8 h. Cell and supernatant were separated by centrifugation at 12,000 rpm, 4˚C for 20 min. The TGase activity was determined using hydroxamate method.

4.4 Results and discussion

4.4.1 Primer designing

The primers obtained from specific primer designing were shown in Table 4.1 and from degenerate primer designing were shown in Table 4.2.

Table 4.1 Primers from specific primer designing.

Primer	Nucleotide sequence
Fw1	5'-CTCGTCTTCGCCACTATGAG-3'
Fw2	5'-CTATGAGTGCGGTGTTATGC-3'
Fw3	5'-AAATTCCTGTGAATTAGCTGATTTAG-3'
Fw4	5'-CAATTGGCTGAAGAAACCGT-3'
Fw5	5'-TACAAGCGTCGGAGTTTACTC-3'
Fw6	5'-TTCACGAAATTCCACAACAAGG -3'
Fw7	5'-CGTTTCACGAAATTCCACAACAAGG -3'
Fw8	5'-TTTCATGTACAAGCGTCGGA-3'
Rw1	5'-GGGATGAAGGTGATCACATAGG-3'
Rw2	5'-GATGGAGAGGGTGATACCAC-3'
Rw3	5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'
Rw4	5'-AAATTGTTTCGTCAGATCGC-3'
Rw5	5'-CCAGCTCTTGGGTATGAACG-3'
Rw6	5'-CCAGCTCTTGGGTATGAACG-3'
Rw7	5'-GTGTTCCAGCTCTTGGGTATGAACG-3'
Rw8	5'-CTTCATACAGGTCCGGATGG-3'

Table 4.2 Primers from degenerate primer designing.

Primer	Nucleotide sequence
tgFw1	5'-ATCTACTAGTGAYGASMGGGWVACYCCTCC-3'
tgFw2	5'-ATCTGGATCCGAYMGGGWVACYCCTCC-3'
tgRw1	5'-TCACGGCCAGCCYTGCTYYAC-3'
tgRw2	5'-AGATCTGCAGCGGCCAGCCYTGCTYYA C-3'

4.4.2 Cloning of TGase gene

The genomic DNA of *P. thailandensis* and *E. siamensis* were amplified by the primers in Table 4.1 and 4.2 at various PCR conditions of both DNA polymerases. It was found that the PCR product from the genomic DNA of *E. siamensis*, which was amplified using F1R1 and F2R1 primer with 2× Taq Master Mix, showed the band as 1100-bp (Figure 4.1a) and 1200-bp (Figure 4.1b), respectively. In addition, the PCR product of *P. thailandensis*, which was amplified by F3R3 with 2×Taq Master Mix, showed the band size of 500-bp (Figure 4.2). However, no band was appeared on agarose gel when gonomic DNA were amplified using F2F2, for both strains. Moreover, the PCR product showed multiple bands when genomic DNA was amplified by tgFtgR2 primer with Takara *Ex Taq* DNA

polymerase (Figure 4.3). For other primers, the amplicons did not appear due to the primers may be not specific with the target gene and PCR conditions were not suitable.

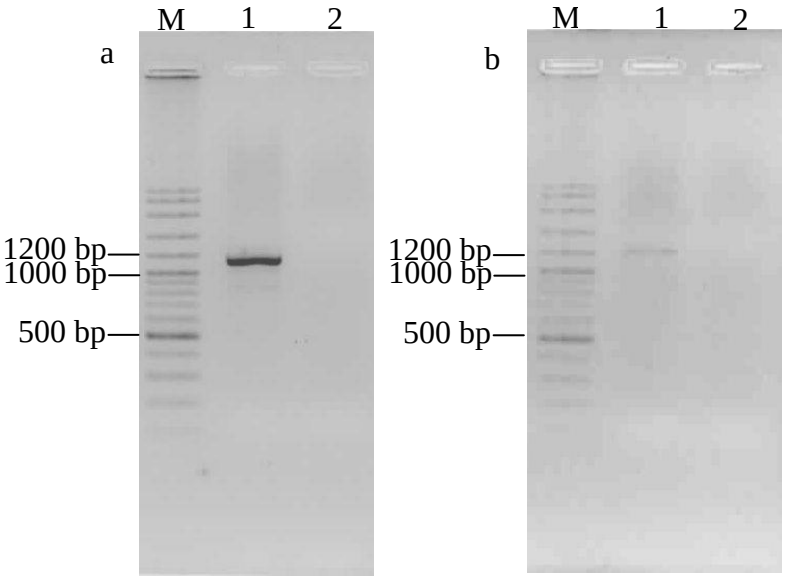


Figure 4.1 Agarose gel electrophoresis of PCR products of TGase gene amplification from *E. siamensis*. 1a and 1b were amplified using F1R1 and F2R1 primers, respectively. Lanes: M, 100 bp Plus DNA Ladder; 1, *E. siamensis*; 2, negative control.

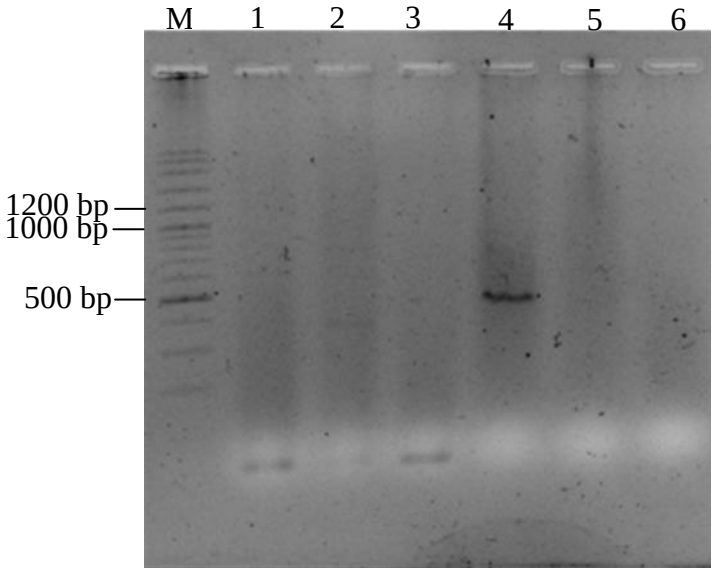


Figure 4.2 Agarose gel electrophoresis of PCR products of TGase gene amplification using F2R2 primers. (lane 1-3) and F3R3 primer (lane 4-6). Lanes: M, 100-bp Plus DNA Ladder r; 1, 4: *P. thailandensis* ; 2, 5: *E. siamensis* ; 3, 6: negative control.

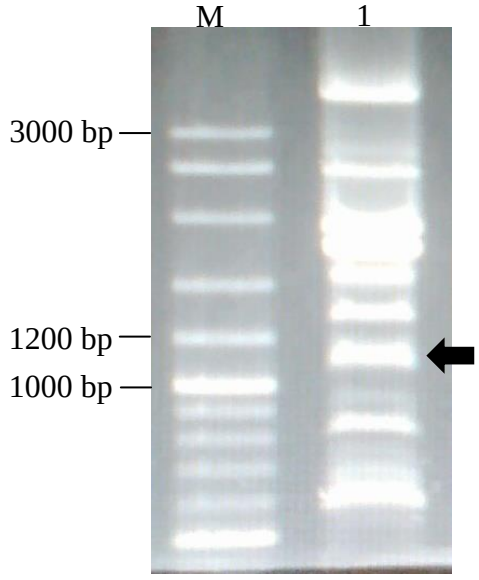


Figure 4.3 Agarose gel electrophoresis of PCR products of TGase gene amplification using tgFtgR2 primer with Takara *Ex Taq* DNA polymerase. Lanes: M, 100-bp Plus DNA Ladder; 1, *P. thailandensis.* Arrow, indicating PCR product was cut to purify and use in gene cloning.

Then PCR products of *E. siamensis*, 1100-bp and 1200-bp PCR products, were sequenced. The sequencing showed 958 and 510 nucleotides, respectively (Figure 4.4 and 4.5). Both nucleotide sequences were translated to amino acids and blasted on GenBank. The results showed 96% similarity to ABC transporter permease (*E. cloacae*) and 71% similarity to hypothetical protein (*Enterobacteriaceae*), respectively.

```
  1 ACATGCATCG CGGGGAGTGT ATGACGTTCT GGAGCATTGT GCGCCAGCGC 50
 50 TGCTGGGGGT TAATCCTGGT AGTAGCCGGT GTCTGTATCA TCACCTTTAT 100
101 CATTTCACAC CTGATCCCCG GCGATCCGGC CCGCCTGCTG GCCGGTGACC 150
151 GTGCCAGCGA CGAGATTGTG CAAAAACATT CGCCAGCAAC TGGGACTGGA 200
201 TCAGCCGCTC TATATCCAGT TTGGCCGCTA TGTGGACGCC CTGGCGCACG 250
251 GCGATTTAGG GACCTCTATC CGTACCGGGC GGCCGGTGGC GGAAGATTTA 300
301 AAAGCCTTCT TCCCTGCCAC GCTGGAGCTG GCGTTCTGTT CCCTGCTGCT 350
351 GGCGCTGGTG ATCGGCGTTC CGCTGGGGAT TTTATCGGCG GTCTACCGTA 400
401 ACCGCTGGCT GGATCATCTG GTACGGCTGA TGGCGATGAC CGGGATCTCC 450
451 ACACCGGCCT TCTGGCTGGG TCTGGGCGTC ATCGTCTTGT TCTACGGCCA 500
501 TCTTCAGCTT CTGCCCGGCG GCGGCAGGCT GGACGACTGG CTCGACCCGC 550
551 CGGCCCACGT GACGGGCTTT TATCTGCTGG ATGCCCTGCT GGAGGGTAAC 600
601 GGCGAGGTCT TTTTCAACGC CCTGCAACAC CTGATTTTAC CGTCACTGAC 650
651 GCTGGCGTTT GTTCATCTTT GGCATCGTGG CGCGTCAGGT ACGTTTCCGC 700
701 CATGCTGGAG CAGCTCAGCG AAGATTACAT TCGCACCGCC CGCGCCAGCG 750
751 GCTTGCCGGG CTGGTATATC GTCCTGCGGT ATGCCCTGCC GAATGCGATG 800
801 ATCCCGTCCA TTACCGTACT CGGGCTGGCG CTGGGCGATC TGCTTTTAGG 850
851 TGCGGTACTG ACCGAAACCG TCTTTGCCTG GCCAGGCATG GGCGCCTGGG 900
901 TGGTGACCTC CATTCAGGCA CTGGATTTCC CTGCCGTCAT GGGCTCGCCG 950
951 TGGTACG                                              958
```

Figure 4.4 Nucleotide of *E. siamensis* sequences TGase gene amplified using F1R1 primer.

```
  1 AAGAAATCTA AAGTAATGTC ATCCAGAATT GTGTTATTCC CATAATGCCC 50
 51 TGTCACAATG CAACGGTGAA ATAACCATCG CCATAACCCT GAAACAGGCA 100
101 TGATACCTTT CGATGATAAT AATGTCAGTT CACCAGAACG AACACCATAA 150
151 GAGGGCAAAA TAATAATTTC CGCATCCTGT CATCCACCTT TTTAAGTCTT 200
201 CTCAGCAGAA ACAAAAGTGG TCTTATATAG ACATCTATAA TAACATCGCT 250
251 TTTCCTGAAA TTAAATTTAA CATGGGTCAT TTCTTCCTTT TCAGAGAGTG 300
300 AGTAAGGAAG TATTTTAATA CAACCAAGGT AATTCCCCCC AATATTAACA 350
351 CCCGGATATA CGGAGCCATG CAAGTATATT CTCAAGGCAT TTTGAATGTA 400
400 GTCATCCACT ACCTGTGACA AAAAATACTT ATTCAGAAGA CGATCGCGCT 450
451 TGTTCTCATC CAGAACATTC AAACTGCTGA CAAAGAACCA GATAGAAAGG 500
501 AAAATATTA                                            510
```

Figure 4.5 Nucleotide of *E. siamensis* sequences TGase gene amplified using F2R1 primer.

The PCR product of *P. thailandensis*, 1100-bp PCR product which amplified using tgFtgR2 primer with Takara *Ex Taq* DNA polymerase (Figure 4.3), was purified from agarose gel and then ligated with pSSBm97 vector. After transformed into *E. coli* 10G, gene insertion were determined and the recombinant DNAs were extracted and digested with *Spe*I. The band obtained were 7.5-kb size (Figure 4.6).

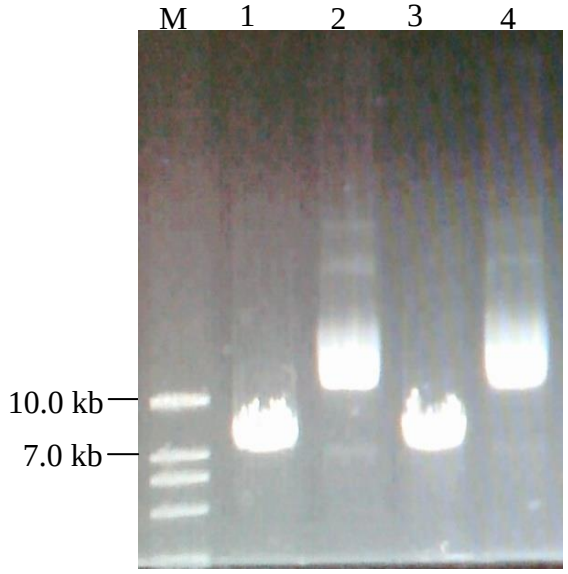


Figure 4.6 The recombinant DNAs were digested with restriction enzyme. Lanes: M, 1-kb ladder DNA marker; 1, 3: Recombinant DNA of colony no.1 and 2 were digested with *Spe*I; 2, 4 recombinant DNA of no.1 and 2 uncut, respectively.

As the PCR efficiency is controlled by many parameters such as polymerase type, buffer type, primer concentration and stability, dNTP purity and concentration, cycling parameters as well as complexity and concentration of the template. Then, it is necessary to optimize PCR conditions for each PCR amplification (Hermann and Foernzler, 2002).

From the sequence of TGase gene searching from GenBank database, there is only TGase gene from *Streptomyces* sp. available. *Streptomyces* sp. is a Gram-positive bacteria while our strains used in this study, *P. thailandensis* and *E. siamensis*, are Gram-negative. Because of TGase gene sequenc needed for primer designing, most of the results from TGase gene amplification showed no band appear

on agarose gel. It may due to different gene conservation between different organism (Tamames, 2001). Kikuchi *et al*. (2011) reported that the *TGase* from *Streptomyces* has high GC content. When TGase gene was amplified in this study, it needed to use suitable DNA polymerase for high GC content amplification. Choosing an appropriate DNA polymerase can be helpful for obtaining desired amplicon products (Lorenz, 2012). Four basic properties of DNA polymerases were defined the best enzyme for particular research needs. First of all, thermal stability, a denaturation step at approximately 95˚C in each PCR cycle separates the two strands of a DNA molecule. DNA polymerase must be robust enough to tolerate high temperature cycles without compromising activity, a factor dependant on buffer composition and pH. Second, extension rate which is refers to the speed at which nucleotides are added per second, per molecule of DNA polymerase. A factor determined by extension temperature, DNA template sequence and buffer composition. Early polymerases exhibited extension rates of about 1 kb per minute at 72˚C but contemporary enzymes are generally faster (approximately 4 kb per minute). Third, fidelity is an inherent DNA polymerase property defining the frequency of insertion of an incorrect nucleotide per kb of DNA. For standard DNA polymerases, fidelity refers to the ability to distinguish correct between incorrect nucleotide incorporation and can be influenced by the buffer composition. High-fidelity DNA polymerases are more accurate because of the ability to "proofread" and excise incorrectly incorporated mononucleotides, replacing them with the correct base. The last, processivity, which a polymerase will unfasten from DNA during extension, indicating the average number of nucleotides the enzyme adds in a single binding event, is known as its processivity. Like extension rate, processivity depends on buffer composition (salt concentrations) and the sequence of a DNA template. High processivity is important when amplification is a long amplicons (Biocompare, 2013).

4.4.3 Expression of pro-TGase

The recombinant DNA from cloning step was subcloned into *B. megaterium* YYBm1 for gene expression. *B. megaterium* YYBm1 carrying pSSBm97-TG was then cultured. However, the activity of TGase could not detect. To solve this problem, the sequencing of pSSBm97-1 has to be determined to check fame of gene insertion. In addition, protein expression needed to try the cultivation

condition such as concentration of the inducer and temperature. It is well known, protein expression at low temperature decrease aggregation, which is favored at higher temperature because the temperature dependence of hydrophobic interactions (Schumann and Ferreira, 2004; Sorensen and Mortensen, 2005).

As TGase gene cloning from *P. thailandensis* and *E. siamensis* were unsuccessful because they were a novel species and Gram-negative, which did not have a data of TGase gene on the GenBank database. Thus, it was difficult to amplify TGase gene since the primers were designed from *Streptomyces* sp. which was Gram-positive. There were quite different gene conservation between Gram-positive and Gram-negative or vary species. Therefore, to succeed for TGase gene cloning part in this thesis, another strain of bacteria was found out and used for further study.

4.4 Conclusion

The PCR products of *E. siamensis* (1100-bp and 1200-bp) were sequenced. The results showed 96% similarity to ABC transporter permease (*E. cloacae*) and 71% similarity to hypothetical protein (*Enterobacteriaceae*) respectively. The PCR product of *P. thailandensis* (1100-bp) was amplified using tgFtgR2 primer with Takara *Ex Taq* DNA polymerase. This PCR product was ligated with pSSBm97, transformed into *E. coli* 10G and then expressed protein into *B. megaterium* YYBm1. However, the activity of TGase could not be detected. Therefore, to succeed for TGase gene cloning part in this thesis, another strain of bacteria was used for further study.

CHAPTER 5

IDENTIFICATION, GENE CLONING AND EXPRESSION OF TRANSGLUTAMINASE GENE FROM *STREPTOMYCES* SP. AH6 IN *ESCHERICHIA COLI*

5.1 Abstract

Transglutaminase (TGase) has been reported to produce by the genus *Streptomyces*, that were mostly isolated from soil. In this study the TGase producing *Streptomyces* was isolated, identified, cloned and expressed in *Escherichia coli*. From the result, the isolate of *Streptomyces*-like strains were obtained and identified using 16S rRNA gene. The stain AH6 showed 99 % similarity with that of *S. thermocarboxydus* and this strain was named as the *Streptomyces* sp. AH6. The *TGase* gene of *Streptomyces* sp. AH6 was constructed by fusion of *TGase* precursor and pET22b plasmid. The recombinant DNAs were transformed into *E. coli* BL21 (DE3). The open reading frame (ORF) TGase of this gene encoding 410 amino acids. Although the amino acids precursor sequence of this *TGase* was 100% homologous to the *TGase* of *S. mobaraensis* NBRC 13476, the nucleotide sequence was different at 6 nucleotides that of T to G (at nucleotide 231); T to C (at nucleotide 234, 237, 240 and 249); and A to C (at nucleotide 246). The pET22b-TG-His_6 was secreted as a soluble inclusion body in *E. coli* and the activity of partial purified enzyme was 3.2 U/ml. The signal peptide and pro-peptide of TGase involved for secretion and solubility of TGase in *E.coli*. The optimal pH and temperature of partially purified TGase were 6.0 and 40˚C respectively. The TGase was completely inhibited by Cu^{2+}, Fe^{2+} and NEM meanwhile PMSF moderately inhibited the activity. Moreover, Ba^{2+}, $Ca2^{2+}$, Co^{2+}, Mg^{2+}, Mn^{2+} and EDTA could decrease the TGase activity.

5.2 Introduction

Transglutaminases (TGases, r-glutaminyl peptide: amine γ-glutaminyl transferase, EC 2.3.2.13) are thiol enzymes that catalyze protein reticulation by introducing isopeptide bonds (ε-(γ -glutaminyl) lysine bonds) (Folk and Finnlayson, 1977). TGase catalyzes an acyl-transfer reaction between the γ-carboxyamide group

of glutamine residue (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine residue in certain proteins. In the absence of amine substrates, TGase catalyzes the deamidation of glutamine residues which water molecules are used as acyl acceptors. TGases can modify proteins by means of amine incorporation, crosslinking and deamidation (Motoki and Seguro, 1998).

Ando and co-worker (1989) reported that strains from the genus *Streptomyces* had the ability to produce TGases as determined by using the hydroxamate assay. These microorganisms screened from several thousand microorganisms excreted the TGase enzyme, and one of them classified as a variant of *S. mobaraensis* (Washizu *et al*., 1994) which produced the highest activity. Microbial TGase is calcium ion-independent and many functional studies demonstrated its industrial application (Kuraishi *et al*., 1996; Motoki and Seguro, 1998; Sakamoto *et al*., 1993; Yokoyama *et al*., 2004). In nature, *Streptomyces* synthesized TGase as a zymogen (pro-TGase), which is processed to produce active enzyme by removal of its N-terminal pro-peptide (Zotzel *et al*., 2003). Then production of TGase active form leads to cell death by cross-linking host proteins (Washizu *et al*., 1994). TGase of *Streptomyces* is usually expressed in the pro-TGase form of heterologous hosts. The pro-TGase is converted into active TGase using addition of activation protease (Yang *et al*., 2009; Liu *et al*., 2015) or coexpressing the protease (Kikuchi *et al*., 2003). The TGase production by genetically modified strains was studied in *Escherichia coli*, *S. lividans* and *Corynebacterium glutaminum*. Lui *et al.* (2011a) reported that the pro-region of TGase is essential for TGase secretion and solubility in *E. coli*.

In this study, *Streptomyces* sp. was isolated from soil at the seafood factory in Songkhla Province, Thailand using a standard dilution plating method and identified as a *Streptomyces* sp. AH6. The TGase gene of *Streptomyces* sp. AH6 was cloned and expressed in *E. coli* BL21 (DE3). The partially purified TGase from gene expression was then characterized.

5.3 Material and Methods

5.3.1 Enzyme assay

The pro-TGase was activated according to slightly modified method of Marx *et al*. (2008). The pro-TGase was activated using trypsin at final concentration of 0.5 µg/µl at 37˚C for 30 min. TGase activity was assayed by hydroxamate method (Folk and Cole, 1966). 50 µl of TGase was mixed with 12 µl of the mixture (174 mM Tris buffer (pH 6.0), 87 mM hydroxylamine, 8.7 mM glutathione, 31 mM CBZ-Gln-Gly and 4 mM calcium chloride). The reaction mixture was incubated at 37˚C for 10 min. Thereafter, 94 µl of 12% TCA solution and 94 µl of $FeCl_3$ in 100 mM HCl was added to the reaction mixture, respectively. After 5 min, the mixture was centrifuged at 10,000×*g* for 5 min and measured the absorbance at 525 nm. One unit of TGase activity was defined as the amount of the enzyme used to produce 1 µmol of hydroxamic acid per min at the assay conditions.

5.3.2 Identification of the selected isolate

5.3.2.1 DNA extraction

The strain AH6 was isolated from soil at Songkhla province. The sample was plated on ISP2 medium (0.4% yeast extract, 1% malt extract, 0.4% dextrose and 2% agar, pH 7.2) and incubated at 30˚C for 3 days. The colonies of *Streptomyces*-like strains were restreaked on ISP4 medium (1% soluble starch, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.2% $(NH_4)_2SO_4$, 0.2% $CaCO_3$ and 0.2% trace salts solution (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$), pH 7.0) and incubated at 30˚C for 7 days. Genomic DNA was prepared by the phenol method of Saito and Miura (1963) with some modifications. Cells were harvested after 7 days of cultivation and suspension in 2 ml of saline-EDTA buffer pH 8.0. Then 100 µl of 10% SDS, 100 µl lysozyme (20 mg/ml), 100 µl protease K (50 mg/ml) solution were added and incubated at 80˚C for 10 min. The phenol extraction was carried out by adding an equal volume of phenol: chloroform (1:1) to the sample. The upper layer of the mixture was collected after centrifugation at 10,000×*g* for 10 min. Genomic DNA was precipitated with two volumes of ice cold absolute ethanol and dried at room temperature. The genomic DNA was dissolved in 1×TE buffer and stored at -20˚C.

5.3.2.2 Sequencing of 16S rRNA gene

Primers ScAct0235aS-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and ScAct0878-aA-19 (5'-CCGTACTCCCCAGGCGGGG-3') were used to amplify 16S ribosomal sequences from the genomic DNA. The amplification reaction was performed with a volume of 25 µl of 2×GoTaq (Promega), 5 µl of each primer (10 pmol) and 1 µl of template DNA (50 ng). Amplification was carried out in a thermocycler with the following cycling program: initial denaturation at 94˚C for 1 min followed by 30 cycles of 94˚C for 30 sec, annealing at 50˚C for 30 sec, extension at 72˚C for 30 sec and a final extension step at 72˚C for 7 min. The PCR product was purified using a PCR purification kit (NucleoSpin Gel and PCR Clean-up). The PCR product was cloned into the T-vector pMD20 (Takara), according to the manufacturer's instructions and transformed into *E. coli* JM109. Plasmid DNA of white colonie was extracted and M13 forward and reverse primers were used to sequence the cloned DNA fragments. The sequencing PCR, the mixture composed of 1.6 µl of each primer (1 pmol/ µl), 100-150 ng of template DNA, 1 µl of premix solution, 1.6 µl of 5×sequencing buffer (BigDye Terminator, Applied Biosystems, Warrington, UK) and sterilized distilled water to 10 µl. The following program was used for this PCR: 96˚C for 4 min, and 25 additional cycles of 96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 4 min. Nucleotide sequencing was done by dideoxy chain termination method with M13 forward and reverse primers using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). Sequences obtained were blasted with the GenBank database.

5.3.3 Primer designing

The primers of TGase gene amplification were designed from the *Streptomyces mobaraensis* NBRC13476 (GenBank accession no. AY762265, Figure 5.1). Primers Fw1: (5'-ATGTCCCAACGCGGGAGTCTCGTCTTCGCCGCTCTC G-3'), was designed from prepro-region of open reading frame (ORF) TGase and Rw1: (5'-TTATGG CCAACCTTGTTTGACCTTGTCGGGGGCGGTGTTC-3') was designed at last 40 nucleotides of the terminal codon of ORF TGase (Figure 5.2). The Fw2 and Rw2 were designed using sequence combination between pET22b sequence and ORF TGase, generating was used for ligation with the plasmid. Primer Fw2: (5'-gaaggagatatacatATGTCCCAACGCGGGAGAACTCTCG-3'), was designed from

pET22b sequences (lower cases) (Figure 5.3a) and 25 nucleotides of prepro-region (Figure 5.3b) and Rw2: (5'-tggtggtgctcgagTGGCCAACCTTGTTTGACCTTG-3'), was designed from pET22b sequences (lower cases) (Figure 5.3a) and 22 nucleotides of ORF TGase without stop codon (TAA) (Figure 5.3b). In order to construct the expression plasmid, the primers for inverse-PCR were designated as Fw3: (5'-atatgt atatctccttcttaaagt-3') and Rw3: (5'-ctcgagcaccaccaccaccaccact-3'), which were designed from pET22b (Figure 5.4). Inverse-PCR with primers was carried out using pET22b as template, generating the plasmid pET22b with T7 promotor but without signal peptide (pelB).

AY762265.1 Streptomyces mobaraensis protein-glutamine gamma glutamyltransferase gene, complete cds

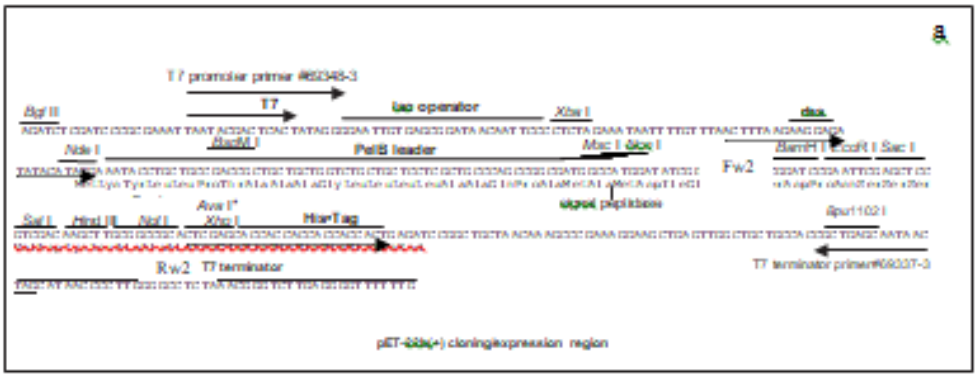
```
   1 ATCCCCATTCCGCCGTGCGGCCGCGGCCTCTTCCTCACCGCCGTTACCGG 50
  51 CGCGGCACCGCAGGACGGGCACCGCCCGACGTTATGCGCGCCACTCGCCG 100
 101 CAACCTCCACCCCCCGCGTCGCACTCTGGCATGCCCTCGTTCCGCGAGGT 150
 151 TCGCCAGATTCAGCCCTTTCGTCACGTTCGCCAAAGGAGTTGTTGTTCTT 200
 201 CATGTCCCAACGCGGGAGAACTCTCGTCTTCGCCGCTCTCGGTGCGGTCA 250
 251 TGTGCACCACCGCGTTAATGCCGTCCGCAGGCGCGGCCACCGGCAGTGGC 300
 301 AGTGGCAGCGGCACCGGGGAAGAGAAGAGGTCCTACGCCGAAACGCACCG 350
 351 CCTGACGGCGGATGACGTCGACGACATCAACGCGCTGAACGAAAGCGCTC 400
 401 CGGCCGCTTCGAGCGCCGGTCCGTCCTTCCGTGCTCCTGATTCAGATGAG 450
 451 CGGGTGACTCCTCCCGCCGAGCCGCTCGACCGGATGCCCGACCCGTACCG 500
 501 GCCCTCGTACGGCAGGGCCGAGACGATCGTCAACAACTACATACGCAAGT 550
 551 GGCAGCAGGTCTACAGCCACCGCGACGGCAGGAAACAGCAGATGACCGAG 600
 601 GAACAGCGGGAGTGGCTGTCCTACGGTTGCGTCGGTGTCACCTGGGTCAA 650
 651 CTCGGGCCAGTATCCGACGAACAGGCTGGCTTTCGCGTTCTTCGACGAGG 700
 701 ACAAGTACAAGAACGAGCTGAAGAACGGCAGGCCCCGGTCCGGCGAAACG 750
 751 CGGGCGGAGTTCGAGGGGCGCGTCGCCAAGGACAGCTTCGACGAGGCGAA 800
 801 GGGGTTCCAGCGGGCGCGTGACGTGGCGTCCGTCATGAACAAGGCCCTGG 850
 851 AGAACGCCCACGACGAGGGGGCGTACCTCGACAACCTCAAGAAGGAGCTG 900
 901 GCGAACGGCAACGACGCCCTGCGGAACGAGGATGCCCGCTCGCCCTTCTA 950
 950 CTCGGCGCTGCGGAACACGCCGTCCTTCAAGGACCGCAACGGCGGCAATC 1000
1001 ACGACCCGTCCAAGATGAAGGCCGTCATCTACTCGAAGCACTTCTGGAGC 1050
1051 GGCCAGGACCGGTCGGGCTCCTCCGACAAGAGGAAGTACGGCGACCCGGA 1100
1101 GGCCTTCCGCCCCGACCGCGGCACCGGCCTGGTCGACATGTCGAGGGACA 1150
1151 GGAACATTCCGCGCAGCCCCACCAGCCCCGGCGAGAGTTTCGTCAATTTC 1200
1201 GACTACGGCTGGTTCGGAGCGCAGACGGAAGCGGACGCCGACAAGACCGT 1250
1251 ATGGACCCACGGCAACCACTACCACGCGCCCAATGGCAGCCTGGGTGCCA 1300
1301 TGCACGTGTACGAGAGCAAGTTCCGCAACTGGTCCGACGGTTACTCGGAC 1350
1351 TTCGACCGCGGAGCCTACGTGGTCACGTTCGTCCCCAAGAGCTGGAACAC 1400
1401 CGCCCCCGACAAGGTCAAACAAGGTTGGCCATAATCTAGAAACAAAAATC 1450
1451 ACTAGTGAATTC                                       1462
```

Figure 5.1 Nucleotide of TGase gene from *S. mobaraensis* NBRC13476. The prepro-region and terminal codon (TAA) were indicated as boldface and underline, respectively.

AY762265.1 Streptomyces mobaraensis protein-glutamine gamma glutamyltransferase gene, complete cds

```
   1 ATCCCCATTCCGCCGTGCGGCCGCGGCCTCTTCCTCACCGCCGTTACCGG 50
  51 CGCGGCACCGCAGGACGGGCACCGCCCGACGTTATGCGCGCCACTCGCCG 100
 101 CAACCTCCACCCCCCGCGTCGCACTCTGGCATGCCCTCGTTCCGCGAGGT 150
 151 TCGCCAGATTCAGCCCTTTCGTCACGTTCGCCAAAGGAGTTGTTGTTCTT 200
 201 CATGTCCCAACGCGGGAGAACTCTCGTCTTCGCCGCTCTCGGTGCGGTCA 250
 251      ACCACCGCGTTAATGCCGTCCGCAGGCGCGGCCACCGGCAGTGGC 300
       Fw1
 301      CAGCGGCACCGGGGAAGAGAAGAGGTCCTACGCCGAAACGCACCG 350
 351 CCTGACGGCGGATGACGTCGACGACATCAACGCGCTGAACGAAAGCGCTC 400
 401 CGGCCGCTTCGAGCGCCGGTCCGTCCTTCCGTGCTCCTGATTCAGATGAG 450
 451 CGGGTGACTCCTCCCGCCGAGCCGCTCGACCGGATGCCCGACCCGTACCG 500
 501 GCCCTCGTACGGCAGGGCCGAGACGATCGTCAACAACTACATACGCAAGT 550
 551 GGCAGCAGGTCTACAGCCACCGCGACGGCAGGAAACAGCAGATGACCGAG 600
 601 GAACAGCGGGAGTGGCTGTCCTACGGTTGCGTCGGTGTCACCTGGGTCAA 650
 651 CTCGGGCCAGTATCCGACGAACAGGCTGGCTTTCGCGTTCTTCGACGAGG 700
 701 ACAAGTACAAGAACGAGCTGAAGAACGGCAGGCCCCGGTCCGGCGAAACG 750
 751 CGGGCGGAGTTCGAGGGGCGCGTCGCCAAGGACAGCTTCGACGAGGCGAA 800
 801 GGGGTTCCAGCGGGCGCGTGACGTGGCGTCCGTCATGAACAAGGCCCTGG 850
 851 AGAACGCCCACGACGAGGGGGCGTACCTCGACAACCTCAAGAAGGAGCTG 900
 901 GCGAACGGCAACGACGCCCTGCGGAACGAGGATGCCCGCTCGCCCTTCTA 950
 950 CTCGGCGCTGCGGAACACGCCGTCCTTCAAGGACCGCAACGGCGGCAATC 1000
1001 ACGACCCGTCCAAGATGAAGGCCGTCATCTACTCGAAGCACTTCTGGAGC 1050
1051 GGCCAGGACCGGTCGGGCTCCTCCGACAAGAGGAAGTACGGCGACCCGGA 1100
1101 GGCCTTCCGCCCCGACCGCGGCACCGGCCTGGTCGACATGTCGAGGGACA 1150
1151 GGAACATTCCGCGCAGCCCCACCAGCCCCGGCGAGAGTTTCGTCAATTTC 1200
1201 GACTACGGCTGGTTCGGAGCGCAGACGGAAGCGGACGCCGACAAGACCGT 1250
1251 ATGGACCCACGGCAACCACTACCACGCGCCCAATGGCAGCCTGGGTGCCA 1300
1301 TGCACGTGTACGAGAGCAAGTTCCGCAACTGGTCCGACGGTTACTCGGAC 1350
1351 TTCGACCGCGGAGCCTACGTGGTCACGTTCGTCCCCAAGAGCTGGAACAC 1400
1401 CGCCCCCGACAAGGTCAAACAAGGTTGGCCATAATCTAG   Rw1   AAATC 1450
1451 ACTAGTGAATTC                                      1462
```

Figure 5.2 Primer Fw1 was designed from prepro-region and Rw1 was designed from terminal codon (TAA) of ORF TGase of *S. mobaraensis* NBRC13476.



AY762265.1 Streptomyces mobaraensis protein-glutamine gamma glutamyltransferase gene, complete cds

b

```
   1 ATCCCCATTCCGCCGTGCGGCCGCGGCCTCTTCCTCACCGCCGTTACCGG 50
  51 CGCGGCACCGCAGGACGGGCACCGCCCGACGTTATGCGCGCCACTCGCCG 100
 101 CAACCTCCACCCCCCGCGTCGCACTCTGGCATGCCCTCGTTCCGCGAGGT 150
 151 TCGCCAGATTCAGCCCTTTCGTCACGTTCGCCAAAGGAGTTGTTGTTCTT 200
 201 CATGTCCCAACGCGGGAGAACTCTCGTCTTCGCCGCTCTCGGTGCGGTCA 250
 251     ACCACCGCGTTAATGCCGTCCGCAGGCGCGGCCACCGGCAGTGGC 300
 301 Fw2 CAGCGGCACCGGGGAAGAGAAGAGGTCCTACGCCGAAACGCACCG 350
 351 CCTGACGGCGGATGACGTCGACGACATCAACGCGCTGAACGAAAGCGCTC 400
 401 CGGCCGCTTCGAGCGCCGGTCCGTCCTTCCGTGCTCCTGATTCAGATGAG 450
 451 CGGGTGACTCCTCCCGCCGAGCCGCTCGACCGGATGCCCGACCCGTACCG 500
 501 GCCCTCGTACGGCAGGGCCGAGACGATCGTCAACAACTACATACGCAAGT 550
 551 GGCAGCAGGTCTACAGCCACCGCGACGGCAGGAAACAGCAGATGACCGAG 600
 601 GAACAGCGGGAGTGGCTGTCCTACGGTTGCGTCGGTGTCACCTGGGTCAA 650
 651 CTCGGGCCAGTATCCGACGAACAGGCTGGCTTTCGCGTTCTTCGACGAGG 700
 701 ACAAGTACAAGAACGAGCTGAAGAACGGCAGGCCCCGGTCCGGCGAAACG 750
 751 CGGGCGGAGTTCGAGGGGCGCGTCGCCAAGGACAGCTTCGACGAGGCGAA 800
 801 GGGGTTCCAGCGGGCGCGTGACGTGGCGTCCGTCATGAACAAGGCCCTGG 850
 851 AGAACGCCCACGACGAGGGGGCGTACCTCGACAACCTCAAGAAGGAGCTG 900
 901 GCGAACGGCAACGACGCCCTGCGGAACGAGGATGCCCGCTCGCCCTTCTA 950
 950 CTCGGCGCTGCGGAACACGCCGTCCTTCAAGGACCGCAACGGCGGCAATC 1000
1001 ACGACCCGTCCAAGATGAAGGCCGTCATCTACTCGAAGCACTTCTGGAGC 1050
1051 GGCCAGGACCGGTCGGGCTCCTCCGACAAGAGGAAGTACGGCGACCCGGA 1100
1101 GGCCTTCCGCCCCGACCGCGGCACCGGCCTGGTCGACATGTCGAGGGACA 1150
1151 GGAACATTCCGCGCAGCCCCACCAGCCCCGGCGAGAGTTTCGTCAATTTC 1200
1201 GACTACGGCTGGTTCGGAGCGCAGACGGAAGCGGACGCCGACAAGACCGT 1250
1251 ATGGACCCACGGCAACCACTACCACGCGCCCAATGGCAGCCTGGGTGCCA 1300
1301 TGCACGTGTACGAGAGCAAGTTCCGCAACTGGTCCGACGGTTACTCGGAC 1350
1351 TTCGACCGCGGAGCCTACGTGGTCACGTTCGTCCCCAAGAGCTGGAACAC 1400
1401 CGCCCCCGACAAGGTCAAACAAGGTTGGCCATAATCTAGAAACAAAAATC 1450
1451 ACTAGTC                                           1462
          Rw2
```

Figure 5.3 Primer Fw2 and Rw2 were designed using sequences combination between pET22b and *S. mobaraensis* NBRC13476. (a) pET22b sequence was showed as the lower cases of Fw2 and Rw2. (b) Nucleotides of ORF TGase were showed as Fw2, which was 25 nucleotides sequences of prepro-region and Rw2, which was the nucleotide sequences without stop codon.

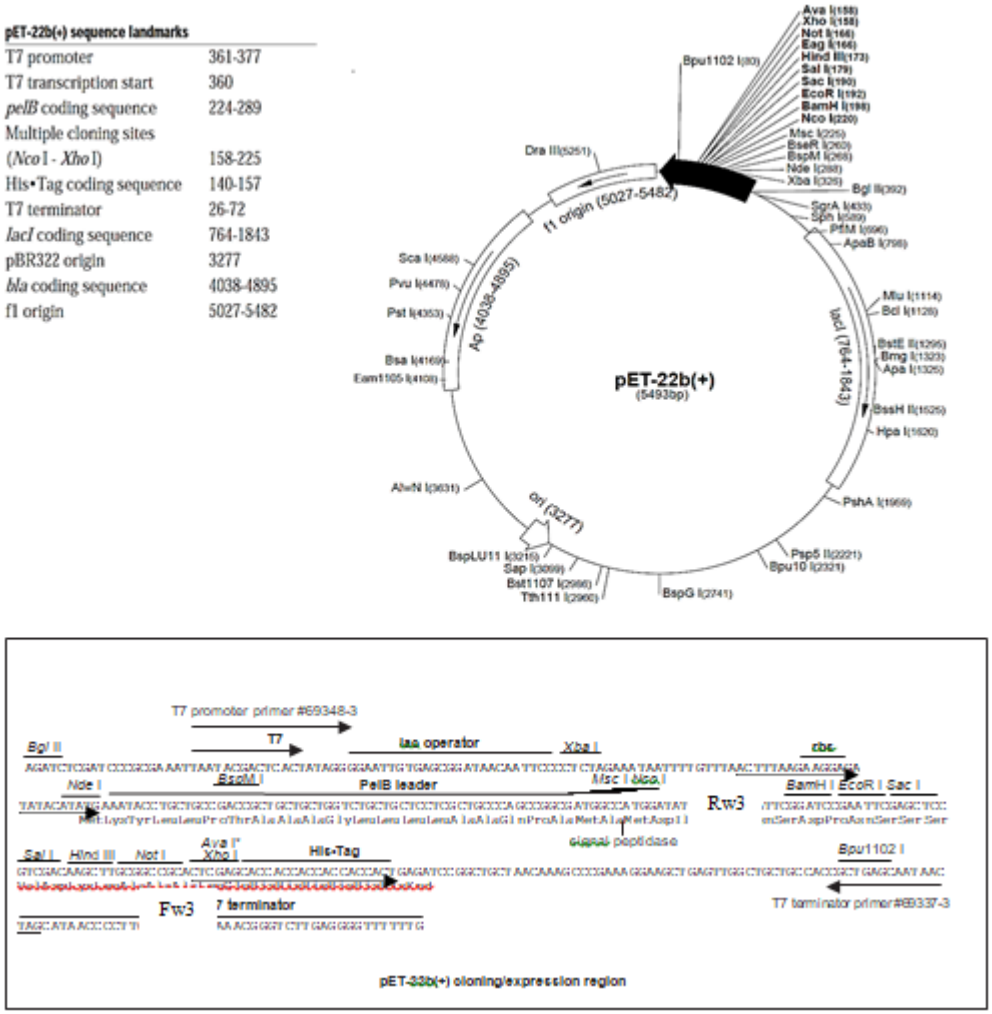


Figure 5.4 Primers Fw3 and Rw3 using for inverse-PCR were designed from pET22b.

5.3.4 Cloning of TGase gene

The genomic DNA from *Streptomyces* sp. AH6 was extracted using a Takara Kit. The TGase gene was amplified from the genomic DNA using primers Fw1 and Rw1. These primers used for amplification of the full ORF TGase gene. The PCR reaction was performed by Takara LA taq DNA polymerase (DNA proof reading polymerase with 3' to 5' exonuclease activity) over 10 min at 95˚C, 30 cycles of 1 min at 94˚C, 45sec at 65˚C, 2.5 min at 72˚C, and a final elongation step of 10 min at 72˚C. The second PCR was performed with primers Fw2 and Rw2. At this step, the primers were used for amplification of ORF TGase without stop codon and it had pET22b sequences, which were used to overlap with the expression plasmid. The PCR reaction was performed using Tks Gflex DNA polymerase (non proof reading DNA polymerase) at 98˚C, 10sec and 68˚C, 1 min for_30 cycles. The pET22b was used as a template for plasmid amplification. The plasmid contained T7 promotor without signal peptide (pelB) was amplified using invert PCR with primers Fw3 and Rw3. The PCR product was treated with DpnI, which was used to digest the plasmid template. The TGase gene was ligated to pET22b with an In-Fusion HD Cloning Kit (TaKaRa Bio Inc.), generating as pET22b-TG-His_6 (Figure 5.5). The ligated product (2.5 µl) was transform into 60 µl of competent *E. coli* JM109. Plasmid DNAs of white colonies were extracted after that T7 promoter and T7 terminator primers were used to sequence the cloned DNA fragments. The method of PCR sequencing, the mixture composed of 1.6 µl of each primer (1 pmol/ µl), 100-150 ng of template DNA, 1 µl of premix solution, 1.6 µl of 5× sequencing buffer (BigDye Terminator, Applied Biosystems, Warrington, UK) and sterilized distilled water to 10 µl. The following program was used for this PCR: 96˚C for 4 min, and 25 additional cycles of 96˚C for 10 sec, 50˚C for 5 sec, and 60˚C for 4 min. Nucleotide sequencing was done by dideoxy chain termination method with M13 forward and reverse primers using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Figure 5.5 Construction of a set of expression plasmid (pET22b-TG-His_6). It consisted of the T7 promoter from pET22b, prepro-region and mature TGase from TGase gene, his_6 tag from pET22b.

5.3.5 Expression and partial purification of pro-TGase in *E. coli*

5.3.5.1 Expression of pro-TGase in *E. coli*

In order to expressed TGase, the recombinant DNAs were transformed into *E.coli* BL21(DE3). To determine the optimal cultivation time for maximal pro-TGase production, the expression strain was cultured in a 5 ml LB medium and 100 µg/ml ampicillin at 37˚C, 200 rpm. After 10 h of incubation, the culture was transfered into a 100 ml shaking flask containing 50 ml LB medium and 100 µg/ml ampicillin at 37˚C, 200 rpm. When the OD_{600} reached 0.8, IPTG was added at a final concentration 0.5 mM and the culture was incubated at 24˚C, 150 rpm. The sample was taken at 2, 4, 6, 8, 10, 12, 14 and 16 h for determination of cell growth and TGase production. The cells and supernatant were separated by centrifugation at 10,000×*g*, 4°C for 20 min. The cells were collected and sonicated in 10 mM KPB (potassium phosphate buffer) pH 7.0. The supernatants were collected and separated by centrifugation at 10,000×*g*, 4°C for 20 min. Protein concentrations were determined using a Bio-Rad protein assay Kit (Hercules, CA, USA) with bovine serum albumin (Wako, Osaka, Japan) as a standard (Bradford, 1976). The enzme was activated by trypsin and TGase activity was determined using hydroxamate method.

5.3.5.2 Partial purification of pro-TGase in *E. coli*

5.3.5.2.1 Production of pro-TGase in *E. coli*

The expression strain was cultured in a 5 ml LB medium and 100 µg/ml ampicillin at 37˚C, 200 rpm. After 10 h of incubation, the culture was transfered into a 100 ml flask containing a 50 ml LB medium and 100 µg/ml ampicillin at 37˚C, 200 rpm. When the OD_{600} reached 0.8, IPTG was added at a final

concentration 0.5 mM and the culture was incubated at 24˚C, 150 rpm for 8 h. The cells and supernatant were separated by centrifugation at 10,000×*g*, 4°C for 20 min. The cells were collected and sonicated in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 25 mM imidazole, pH 7.4). The supernatant from sonication was the intracellular soluble fraction and it was partially purified by nickel affinity chromatography (Ni Sepharose™ 6 Fast Flow, GE Healthcare). The cell debris from the sonication is the intracellular insoluble fraction. All fractions were analyzed using SDS-PAGE.

5.3.5.2.2 Affinity chromatography

Histidine-tagged was fused at C-terminus of TGase gene, pro-TGase expressed in *E. coli* was purified by Ni Sepharose 6 Fast Flow. Briefly, protein concentration of pro-TGase was measured for bed volume calculation to pack the column. Binding capacity of Ni Sepharose 6 Fast Flow was approximately 40 mg/ml. Bed volume was calculated using the formula (GE Healthcare, 2010):

$$\text{Bed volume (L)} = \frac{\text{Bed height (cm)} * \text{Column crossectional area (cm}^2\text{)}}{1000}$$

The sample was loaded onto the column after that the column was washed with binding buffer. The proteins were eluted with the elution buffer which was carried out at 500 mM imidazole. The fractions were assayed TGase activity.

5.3.5.2.3 Purification of the active TGase using Affinity chromatography

In order to obtain the active TGase, the positive fractions from affinity chromatography were dialyzed against 10 mM KPB pH 7.0 at 0˚C, overnight. The dialysate was activated by trypsin at 37˚C for 30 min. The mixture was applied to Ni Sepharose 6 Fast Flow column, and then the column was washed with binding buffer. The proteins were eluted with the elution buffer which contained 500 mM imidazole. The fractions were assayed TGase activity. The active fractions were dialyzed against 10 mM KPB pH 7.0 at 0˚C, overnight. The obtained enzyme was assayed for TGase activity and analyzed by SDS-PAGE.

5.3.5.2.4 SDS-PAGE

The mixtures were determined by SDS-PAGE according to the method of Laemmli (1970) using stacking and separating gels of 4% and 10%

polyacrylamide, respectively. Samples were denatured in SDS sample buffer (10% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.125 M Tris–HCl, pH 6.8) and incubated at 100˚C for 10 min. Gel was run at 50 volt for 30 min and 100 volt for 60 min, stained by Coomassie Brilliant Blue R-250 and destained using destaining solution. The destained gels were visualized by Gel Doc (Gel Doc™ EZ Gel Documentation System, Bio-Rad Laboratories, Hercules, CA, USA).

5.3.6 Characterization of the TGase

5.3.6.1 The effect of pH on the activity and stability

The optimal pHs of partially purified TGase activity was determined with various buffers: a 1000 mM sodium citrate buffer (pH 3.0-7.0), a 1000 mM, phosphate buffer (pH 6.0-7.0), a 1000 mM Tris-HCl (pH 7.0-9.0) and a 1000 mM Glycine-NaOH (pH 9.0-11.0). The TGase activity was assayed at 37˚C for 10 min. The stability of TGase at various pHs were studied using same buffers at 50 mM. The TGase activity was measured after 60 min at 37˚C under TGase assay condition (Folk and Cole, 1966).

5.3.6.2 The effect of temperature and stability

The effect of temperature on the TGase activity was tested by assaying the activity at different temperatures in the range of 0-100˚C for 10 min at pH 6.0. For determination of temperature stability, the partially purified TGase was incubated at different temperature of 0-100˚C for 60 min. After that the residual activity was assayed.

5.3.6.3 Effect of metal ions on TGase activity

The effects of metals ions ($MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $BaCl_2 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $ZnCl_2$ and $FeCl_2 \cdot 4H_2O$) were determined by adding them at final concentration of 5 mM to the reaction mixture and incubating at 37˚C for 30 min. After that the TGase activity was assayed.

5.3.6.4 Effect of inhibitors on TGase activity

The enzyme inhibitors used for testing were ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and N-ethylmaleimide (NEM). The enzyme was mixed with each inhibitor at final concentration of 1 mM and the

mixture was incubated for 30 min at 37˚C and then the residual TGase activity was determined.

5.4 Results and Discussion

5.4.1 Identification of expected genus of the selected strain

The strain AH6 was identified by 16S rRNA gene. The BLAST search results for the 16S rRNA gene partial sequences of the isolate indicated that it had 99 % similarity with that of *Streptomyces thermocarboxydus*. Then the strain AH6 was called as the *Streptomyces* sp. AH6.

5.4.2 Cloning of the TGase gene

The previous reports indicated that strains from genus *Streptomyces* had the ability to produce TGase. Therefore, *Streptomyces* sp. AH6 was used to study in gene cloning step. Although *Streptomyces* sp. AH6 wild strain could not be detected for TGase activity, the TGase precursor of this stain was amplified, cloned and expressed in *E. coli* for improving the production of TGase enzyme. To amplify TGase gene of *Streptomyces* sp. AH6, the primers were designed from *S. ladakanum* BCRC12422 (*S. mobaraensis* NBRC 13476) (Tzeng *et al*., 2005). The TGase precursor (a pre-region, a pro-region and the mature *TGase* was inserted into pET22b plasmid (Lui *et al*., 2011). In addition, T7 promoter was added at N-terminal of TGase precursor and a His_6-tag was added at C-terminus to facilitate protein purification. The pET22b plasmid has T7 promoter and an inducible T7 expression system is highly effective and widely used to produce RNAs and proteins from cloned coding sequences in *E. coli*. The coding sequence for T7 RNA polymerase is present in the chromosome under control of the inducible *lac*UV5 promoter in hosts such as BL21(DE3). The coding sequence for the desired protein (target protein) is placed in a plasmid under control of a T7 promoter, that is, a promoter recognized specifically by T7 RNA polymerase. In the absence of induction of the *lac*UV5 promoter, little T7 RNA polymerase or target protein should be present and the cells should grow well. However, when addition of an inducer (IPTG), T7 RNA polymerase will be made and will transcribe almost any DNA controlled by the T7 promoter. T7 RNA polymerase is so specific, active, and processive, which the amount of target RNA produced can be comparable to the amount of ribosomal RNA in a cell. If the target RNA contains a

coding sequence with appropriate translation initiation signals (such as the sequence upstream of the start codon for the T7 major capsid protein), most protein synthesis will be directed toward target protein, which usually accumulates to become a substantial fraction of total cell protein (Novagen, 2003; Studier, 2005).

The TGase gene with flanking region in this study was called pET22b-TG-His_6. The nucleotides were obtained from TGase precursor gene cloning, which was analyzed ORF by GenTeX software, and showed the ORF TGase encoding 410 amino acids. It started with a methionine codon, six histidines and stop codon (TGA) (Figure 5.6). The active site of the TGase of *Streptomyces* sp. AH6 was YGCVG at amino acid residue 141-145. The sequence was similar to the active site sequences of *S. mobaraensis* NBRC 13476 (assession no. AY762265) (Tzeng *et al*., 2005) *S. cinnamoneus* (assession no. AB085698), *S. fradiae* (assession no. DQ432028), *S. hygroscopicus* (assession no. HM231108) *S. platensis* (assession no. AB451018) (Figure 5.7) and *S. ladakanum* B1 (Lin *et al*., 2004). The amino acids precursor sequence of the TGase from *Strptomyces* sp. AH6 was 100% homologous to the TGase of *S. mobaraensis* NBRC 13476. However, the the nucleotide sequence was different at 6 nucleotides which were T to G (at nucleotide 231); T to C (at nucleotide 234, 237, 240 and 249) and A to C (at nucleotide 246) (Figure 5.8).

The three-dimensional structure of TGase was studied by Kashiwagi *et al*. (2002). The *S. mobaraense* TGase was constructed as a plate-like structure. There were two cysteine residues at amino acids 18 and 143 observed in the TGase from *S. mobaraensis* (NBRC 13476). The former one was in prepro-region and the later was located in the mature enzyme. Therefore, Cys present in the mature TGase could play a specific role in the catalysis of acyl transfer reaction. In addition to the active site region, the active cleft could also play a crucial role to the catalysis. The crystal structure of *S. mobaraense* TGase illustrated that the active cleft comprising the catalytic triad, Cys64-Asp255-His274, superimposed well with that of factor XIII TGase (Yee *et al*., 1994) and sea bream TGase (Noguchi *et al*., 2001). On the other hand, Pasternack *et al*. (1998) found that TGase from *Streptomyces* was secreted as a pro-TGase and could be activated by several exogenous protease. Next, the proteinases participated in the activation of pro-TGase had recently been purified and characterized (Zotzel *et al*., 2003). The endogenous metalloprotease truncated the pro-

domain and generated_the TGase (FRAP-TGase) identical to that activated by *B. polymyxa* dispase. Moreover, the extra tetrapeptide could be removed with the cleavage of a tripeptidyl aminopeptidase. Zotzel *et al*. (2003) revealed that the proteolytic digestion of bovine chymotrypsin could liberate the RAP-TGase from the strain s-811 2 pro-enzyme. The result suggested that the little difference occurred at the N-terminus of active TGase could be due to the actions of several proteinases and these variants still retained the efficient catalysis (Date *et al*. , 2003; Kikuchi *et al*., 2003).

Figure 5.6 Nucleotide and amino acid sequences of the ORF TGase from *Strptomyces* sp. AH6. The prepro region, active site and his_6 tag were indicated as underline, double-underline and box, respectively.

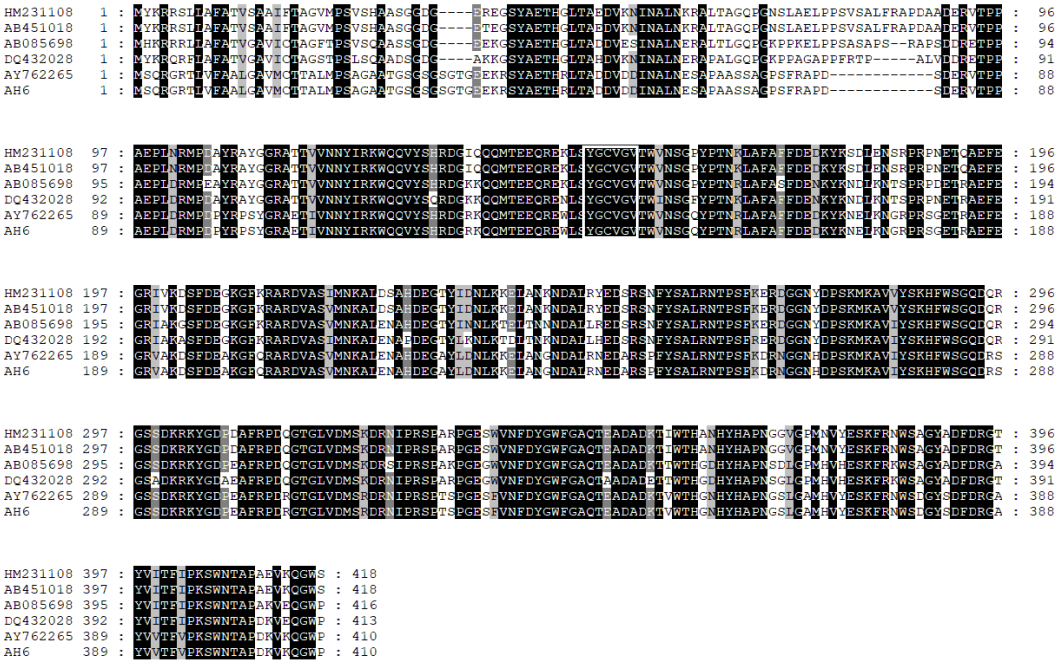


Figure 5.7 Alignment of amino acids of ORF TGase gene from *Streptomyces* species. GenBank accession nos. (left side) AB085698; *S. cinnamoneus*, DQ432028; *S. fradiae*, HM231108; *S. hygroscopicus*, AY762265; *S. mobaraensis*, AB451018; *S. platensis* and AH6; *Streptomyces* sp. AH6. The active site region was boxed.

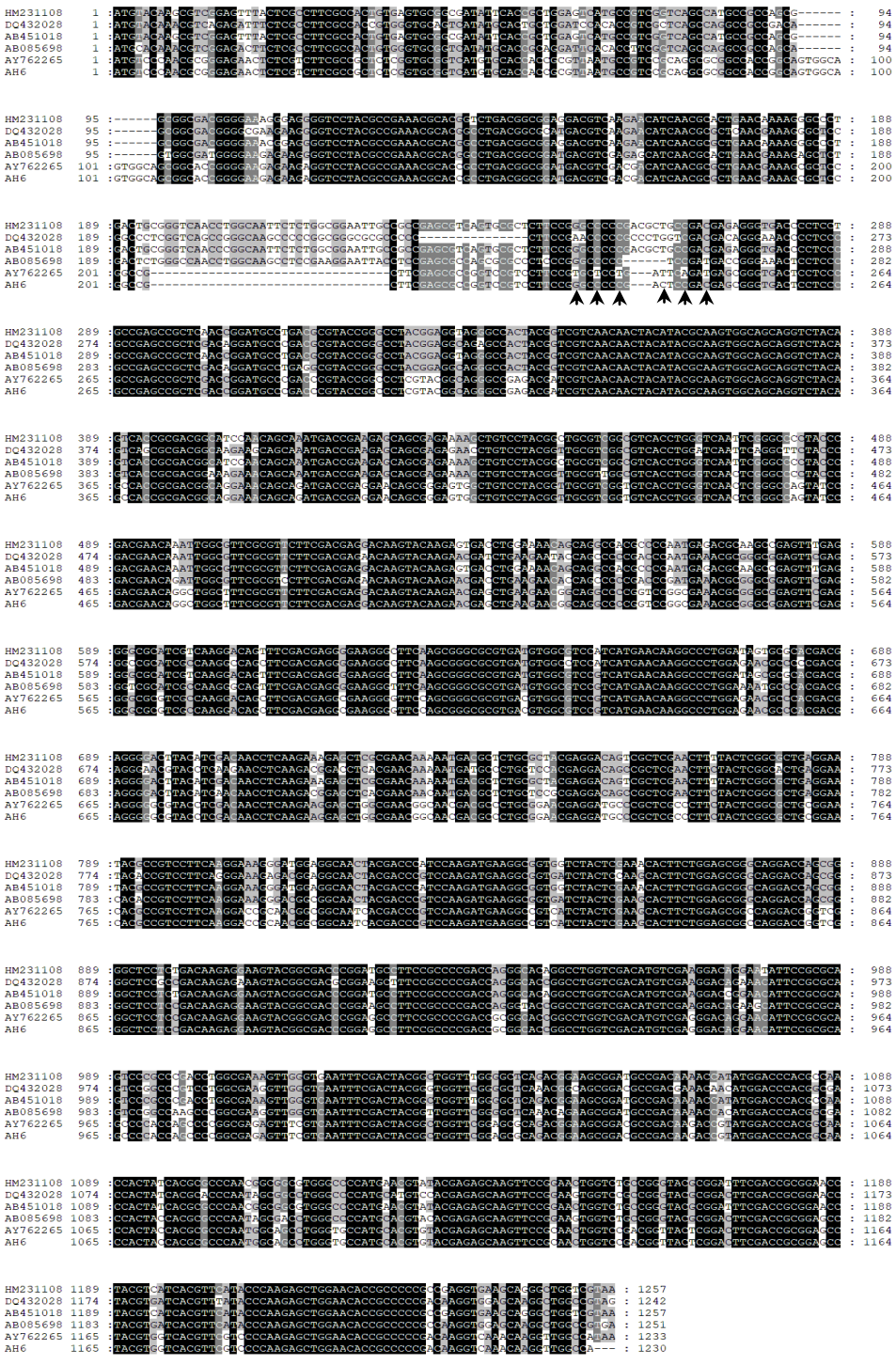


Figure 5.8 Alignment nucleotide of ORF TGase gene from *Streptomyces* species. GenBank accession nos. (left side) AB085698; *S. cinnamoneus*, DQ432028; *S. fradiae*, HM231108; *S. hygroscopicus*, AY762265; *S. mobaraensis*, AB451018; *S. platensis*. The nucleotides of *Streptomyces* sp. AH6 were different from *S. mobaraensis* NBRC 13476, that were indicated by the arrow.

5.4.3 Expression and partial purification of the pro-TGase in *E. coli*

In order to express pro-TGase, it was transformed into *E.coli* BL21 (DE3). The expression strain was cultured at 37˚C until the optical density at 600 nm reached 0.8. After that, the temperature was reduced to 24˚C and IPTG was added. The highest level of productivity of TGase was 8 h at 24˚C and 150 rpm, during the mid-log phase of cell growth after induction (Figure 5.9). However, TGase activity was decreased after 8 h of induction and decrease afterward. The long incubation of cultures for many hours after induction usually affected on the solubility or level of target protein (Studier, 2005).

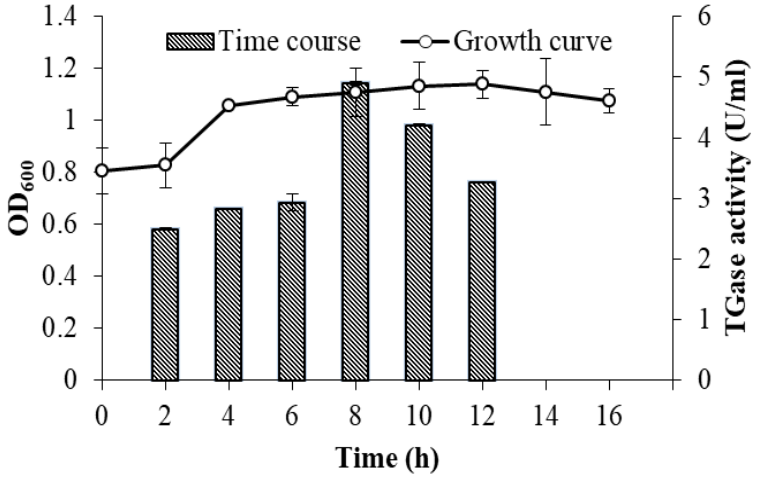


Figure 5.9 Growth and TGase production by recombinant *E. coli* BL21(DE3) with expression of pET22b-TG-His_6 at 24˚C, 150 rpm in LB medium.

The pET22b-TG-His_6 was secreted as soluble inclusion body in *E. coli*. The cells were sonicated and the soluble fractions of recombinant cell lysis were partially purified using Ni Sepharose 6 Fast Flow. Additional, pro-TGase, which obtained from partially purified was activated by trypsin and purified using Ni Sepharose 6 Fast Flow for determination of active TGase form. The supernatant from sonication was the intracellular soluble fraction and the cell debris from the sonication was the intracellular insoluble fraction. The results on SDS-PAGE (Figure 5.10) showed protein expression of pET22b-TG-His_6 after induction at 24˚C. It produced the soluble and insoluble fraction (lane 4 and 5). The partially purified pro-TGase

from purification by Ni Sepharose 6 Fast Flow was estimated to be 45 kDa (lane 7), which was similar to the TGase of *S. hygroscopicus* (Lui *et al*., 2011) and the molecular mass of the active TGase fractions were approximately 38 and 27 kDa (lane 8). The prepro-region of TGase may be digested at arginine residue (at 77 amino acid position), which showed active TGase about 38 kDa. This result was nearly the

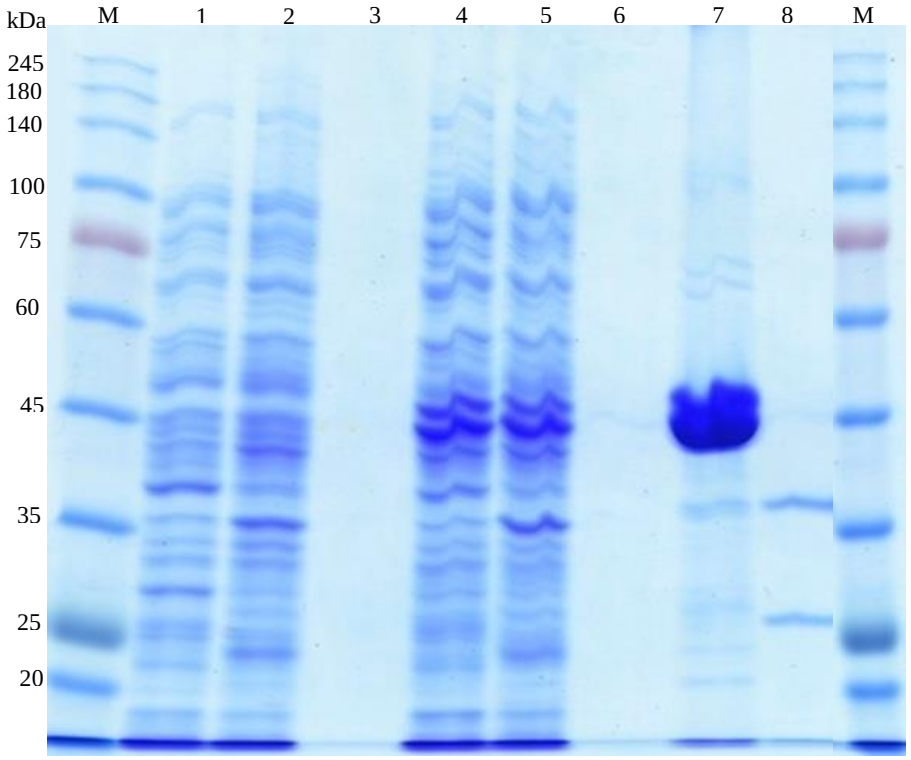


Figure 5.10 SDS-PAGE analysis of expressed pro-TGase in *E. coli* BL21(DE3). Lane M: protein marker; 1, 2, 3: soluble, insoluble and cell-free supernatant of *E. coli* BL21 (DE3) without IPTG; 4, 5, 6: soluble, insoluble and cell-free supernatant of *E. coli* BL21 (DE3) with IPTG; 7: fraction of partially purified TGase using nickel affinity chromatography and 8: fraction of partially purified TGase was digested with trypsin and purified using nickel affinity chromatography.

same as the mature TGase from *S. mobaraensis* NBRC 13476 (Tzeng *et al*., 2005). Another one, the molecular mass of the active TGase was 27 kDa, it may be digested at the lysine residue (at 139 amino acid position).

The molecular mass of the mature TGase (active TGase) must be confirmed by the future study by using gel filtration technique or mass spectrometry.

The TGase activity of this recombinant presented 3.2 U/ml after purification by affinity column, the insoluble fraction had a very low acivity and it could not detected in the cell-free supernatant. However, the recombinant of *S. mobaeaensis*, which was constructed with the pelB, displayed the specific activity of 14 U/mg (Marx *et al*., 2007). The recombinants pro-TGase, which constructed using insertion the pelB signal peptide or TGase signal peptide of N-terminal of pro-peptide and TGase. The result revealed the secretion of two recombinant strains were extacellular pro-TGase at 20˚C and 25˚C (induction temperature). These results indicate that the pro-region of TGase is essential for its efficient secretion and solubility in *E. coli*. Insertion of pelB signal peptide before the pro-peptide may help the proper folding of the TGase in *E. coli* (Lui *et al*., 2011).

5.4.4 Characterization of the TGase

5.4.4.1 The effect of pH on the activity and stability

The TGase activity was studied between pH 3.0-11.0 at 37˚C. The result showed that the enzyme activity was detected at pH 3.0-11.0 (Figure 5.11). The highest activity of this enzyme was at pH 6.0, which was nearly the same as the TGase from *S. hygroscopicus* (Cui *et al*., 2007). Moreover, it showed relative activity more than 58% in the range of pH values 4.0-11.0. In addition, this enzyme showed pH stability at 37˚C for 60 min around pH 4.0-7.0 with the relative activity remaining >49% (Figure 5.12).

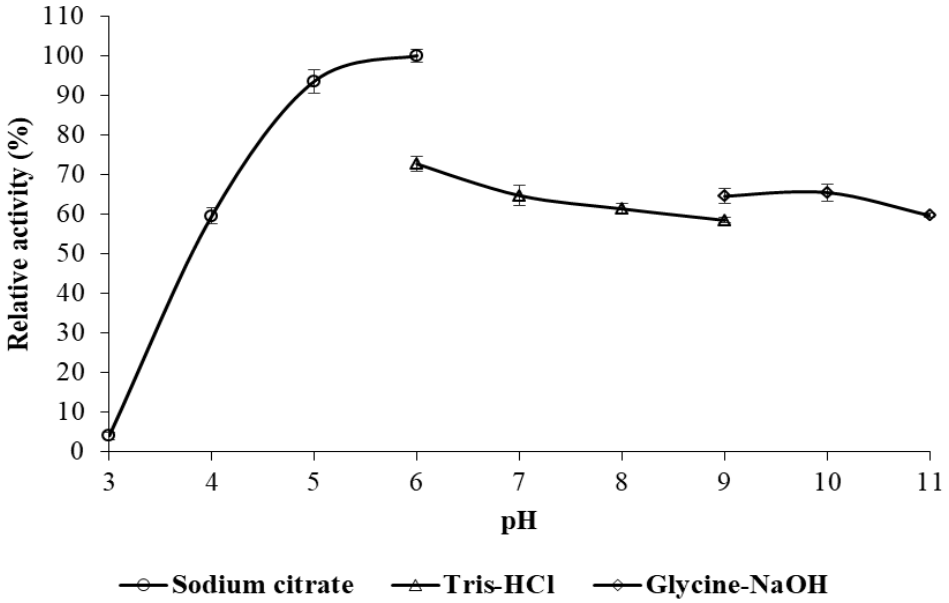


Figure 5.11 Effect of pH on the enzyme activity of partially purified TGase from pET22b-TG-His_6 in *E.coli* BL21(DE3). The buffers used for studing effect of pH were: sodium citrate (pH 3.0-7.0), Tris-HCl (pH 6.0-9.0), and glycine-NaOH (pH 9.0-11.0). The TGase activity was assayed at 37˚C for 10 min.

Kieliszek and Misiewicz (2014) reported that the TGase was produced by bacteria, which were stable at wide range of pH (4.5-8.0). All enzymes have an optimum pH that is the pH at which they work the fastest. However, the pH of a solution can have several effects of the structure and activity of enzymes. For example, pH can have an effect of the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the three dimensional shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive. The changing of pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis (Butler, 1694; Nielsen and Mccammon, 2003).

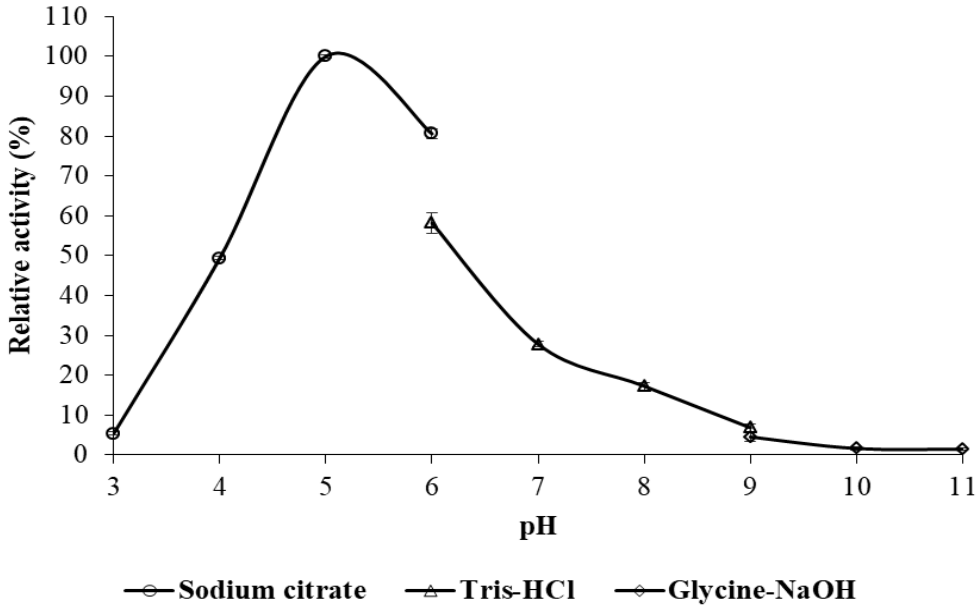


Figure 5.12 Effect of pH on the enzyme stability of partially purified TGase from pET22b-TG-His_6 in *E. coli* BL21(DE3). The buffers used were: sodium citrate (pH 3.0-7.0), Tris-HCl (pH 6 .0-9.0), and glycine-NaOH (pH 9.0-11.0). The partially purified TGase was pre-incubated with various buffers at 37˚C for 60 min.

5.4.4.2 The effect of temperature and stability

The effect use of temperature on the activity of the partially purified TGase was studied by assaying the activity at 0-100˚C. The optimal temperature for the TGase activity was 40˚C. When the temperature was raised above 40˚C, the activity was declined rapidly (Figure 5.13). The thermal stability of TGase was also studied at 0-100˚C. The TGase activity revealed high stability at 0 to 20˚C and it remained activity of >70% at 30˚C for 60 min, while the TGase activity was completely inactivated at 50˚C (Figure 5.14). Comparing with other TGase from different sources, the optimal temperature of TGase of *Streptomyces* sp. and *S. hygroscopicus* was about 40˚C (Macedo *et al*., 2010; Cui *et al*., 2007).

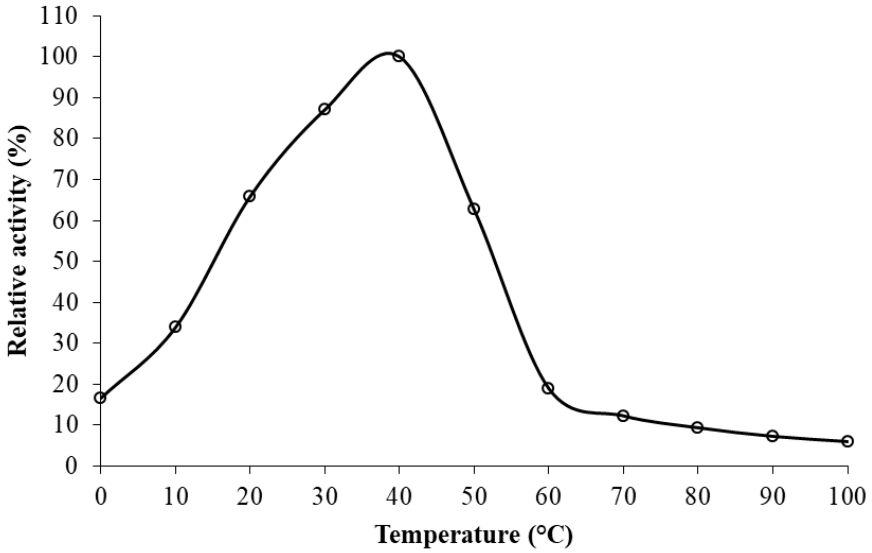


Figure 5.13 Effect of temperature on the enzyme activity of partially purified TGase from pET22b-TG-His_6 in *E. coli* BL21(DE3).

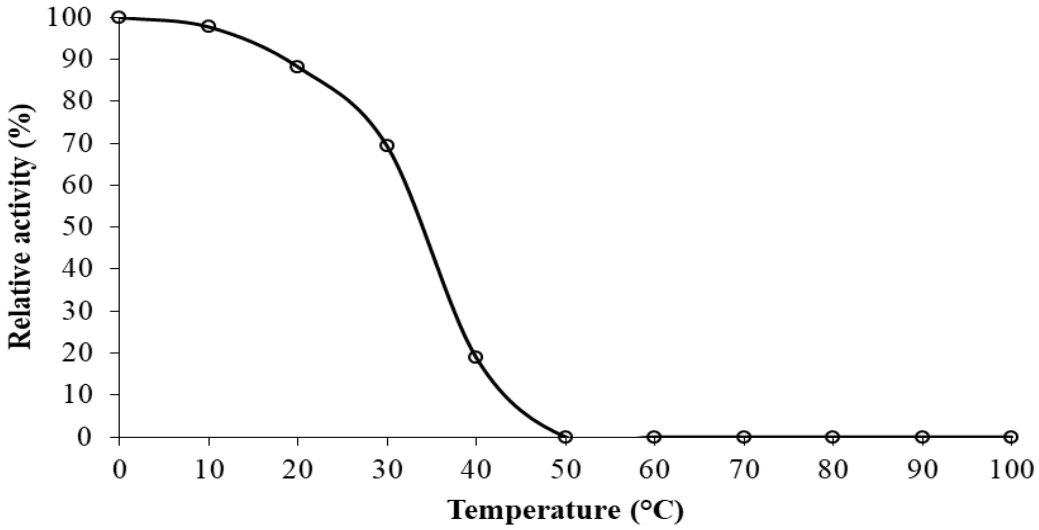


Figure 5.14 Effect of temperature on the enzyme stability of partially purified TGase from pET22b-TG-His_6 in *E.coli* BL21(DE3). The partially purified TGase was pre-incubated different temperatures for 60 min.

5.4.4.3 Effect of metal ions on TGase activity

The activity of the TGase was determined in the reaction mixture containing each metal ion. The TGase activity was completely inhibited by Cu^{2+}, Fe^{2+} and Zn^{2+} meanwhile Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} decreased partial TGase activity (Figure 5.15).

The purified TGases from *S. hygroscopicus* and *Streptomyces* sp. were strongly inhibited by Cu^{2+}, Zn^{2+} and Fe^{3+} (Cui *et al*., 2007, Macedo *et al*., 2010). The Cu^{2+} had strong affinity toward sulfhydryl groups at the active site of TGase (Nozawa *et al*., 1997). While cysteine residues can display high affinity toward Zn^{2+}, and these resulting Zn^{2+}-cysteine complexes are critical mediators of protein structure, catalysis and regulation (Pace and Weerapana, 2014). In addition, Fe^{2+} and Fe^{3+} ion were cofactors, which are often bound by dipole interactions with histidine and other amino acids with lone-pairs (Karami *et al*., 2009).

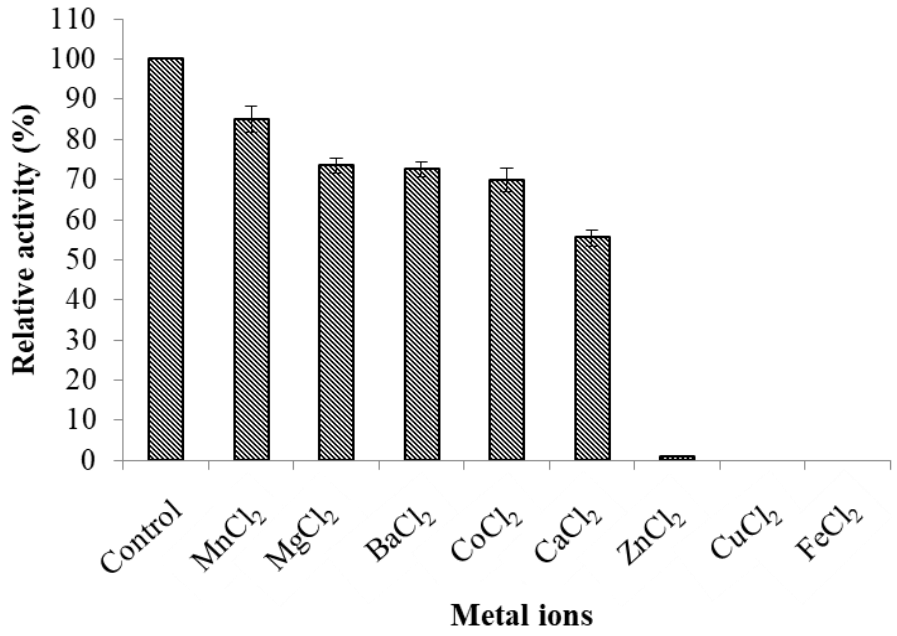


Figure 5.15 Effect of metal ions on the enzyme activity of partially purified TGase from pET22b-TG-His_6 in *E.coli* BL21(DE3). The partially purified TGase was pre-incubated with metal ions at final concentration 5 mM at 37˚C for 30 min.

5.4.4.4 Effect of inhibitors on TGase activity

The relative activity was determined in the enzyme mixture with the additive of inhibitor at final concentration of 1 mM. The TGase activity was completely inhibited by NEM, and was moderately inhibited by PMSF. However, it was not inhibited by EDTA (Figure 5.16). The results of EDTA and NEM were similar to purified TGase activity from *Streptoverticillium* S-8112 (Ando *et al*., 1989). The NEM inhibited TGase activity, indicating that there was a thiol group in its active site. Microbial TGase was not influenced by Ca^{2+}, so the presence of metal chelating compounds such as EDTA could not inhibit enzyme activity (Lin *et al*., 2008). The activity of the partially purified TGase was inhibited partially by PMSF indicating that the active site may be contained serine (Susanti, 2003; Zilda, 2013).

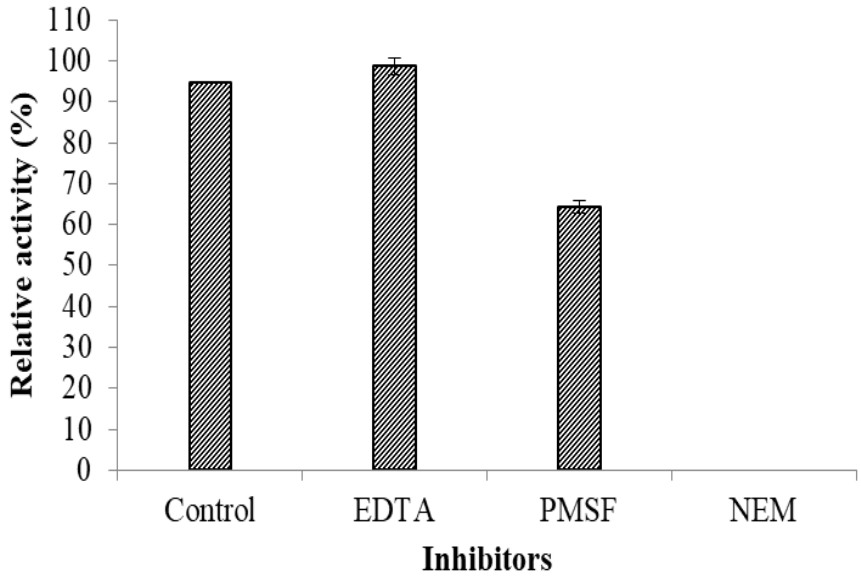


Figure 5.16 Effect of inhibitors on the enzyme activity of partially purified TGase from pET22b-TG-His_6 in *E. coli* BL21(DE3). The partially purified TGase was preincubated with inhibitors at final concentration 1 mM at 37˚C for 30 min.

5.5 Conclusion

The pET22b-TG-His_6 was constructed using fusion of TGase precursor and pET22b plasmid. The T7 promoter was added at N-terminal of TGase precursor and a His_6-tag was added at C-terminus. The ORF TGase encording 410 amino acids. The amino acids precursor sequence of this TGase was 100% homologous to the

TGase of *S. mobaraensis* NBRC 13476, the nucleotide sequence was different at 6 nucleotides that of T to G (at nucleotide 231); T to C (at nucleotide 234, 237, 240, 249); A to C (at nucleotide 246). The pET22b-TG-His6 was secreted as soluble inclusion body in *E. coli*. The partially purified pro-TGase was activated using trypsin at a final concentration 0.5 µg/µl and the activity of partially purified enzyme was 3.2 U/ml. The optimal pH and temperature of partially purified TGase were 6.0 and 40˚C respectively. The TGase activity was completely inhibited by Cu^{2+}, $Fe^{2+}$and Zn^{2+} meanwhile Ba^{2+}, $Ca2^{2+}$, Co^{2+}, Mg^{2+} and Mn^{2+} reduced the activity. The TGase was completely inhibited by NEM, not inhibited by EDTA but the activity was moderately inhibited by PMSF.

CHAPTER 6

SUMMARY AND SUGGESTIONS

6.1 Summary

Providencia thailandensis sp. nov. is proposed. The type strain is C1112^T (= KCTC 23281^T =NBRC 106720^T). They are creamy white, moist, opaque, flatted and undulate margin with 12-19 mm in diameter after 48 h incubation at 37 ˚C on nutrient agar. Cells are Gram-negative, non-spore-forming, rod-shaped and motile with peritrichous flagell. Growth occurs at 15˚C and 45˚C; optimal growth temperature is 30-37˚C. Growth occurs between pH 4.0-12.5 and optimal pH is 6.0-7.0. Growth occurs in the presence of 0-5% (w/v) NaCl and optimally with 0% NaCl.

Enterobacter siamensis are Gram-negative, facultative anaerobic, rod-shaped and motile with peritrichous flagella. Colonies on nutrient agar are creamy white, mucoid, translucent, raised, entire margin, and 5-9 mm in diameter after 48 h incubation at 37˚C. The DNA G+C content is 53.0 mol%. Major cellular fatty acids are $C_{16:0}$, $C_{17:0}$cyclo and $C_{14:0}$. The type strain is C2361^T (KCTC 23282^T, NBRC 107138^T).

PCR products of *E. siamensis*, which amplified using F1R1 and F2R1 primer with 2× Taq Master Mix. They showed 96 and 71% sequence similarity to ABC transporter permease of *E. cloacae* and hypothetical protein of *Enterobacteriaceae*, respectively. While PCR product from *P. thailandensis* was ligated with pSSBm97 and expressed into *B. megaterium* YYBm1. It showed that TGase activity could not detect from expressed protein.

The pET22b-TG-His_6 was constructed using fusion of TGase precursor from *Streptomyces* sp. AH6 and pET22b plasmid. The T7 promoter was added at N-terminal of TGase precursor and a His_6-tag was added at C-terminus. The TGase ORF encording 410 amino acids. The amino acids precursor sequence of this TGase was 100% homologous to the TGase of S. *mobaraensis* NBRC 13476, the nucleotide sequence was were different at 6 nucleotides that of T to G (at nucleotide 231); T to C (at nucleotide 234, 237, 240, 249); A to C (at nucleotide 246). The pET22b-TG-His6 was secreted as soluble inclusion body in *E. coli*. The partial purified pro-TGase was

activated using trypsin at a final concentration 0.5 µg/µl and the activity of partial purified enzyme was 3.2 U/ml. The optimum pH and temperature of partial purified TGase were 6.5 and 40ºC respectively. The TGase was completely inhibited by Cu^{2+} and Fe^{2+} meanwhile it was not inhibited by Ba^{2+}, $Ca2^{2+}$, Co^{2+}, Mg^{2+} and Mn^{2+}. The TGase was completely inhibited by NEM, not inhibited by EDTA and EGTA but the activity was moderately inhibited by PMSF.

6.2 Suggestions

6.2.1 The pET22b-TG-His_6 recombinant DNA should be improved enzyme production using expression in *Streptomyces* sp.

6.2.2 The pET22b-TG-His_6 in *E.coli* BL21(DE3) should be optimized with auto-induction media.

6.2.3 The molecular weigh of TGase from pET22b-TG-His_6 should be studied by using mass spectrometer.

6.2.4 To study the method for removing the trypsin after activation of pro-TGase.

REFERENCES

Aeschlimann, D. and Paulsson, M. 1994. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. Thromb. Haemost. 71: 402-415.

Ahhmedm, A. M., Nasu, T., Huy, D. Q., Tomisaka, Y., Kawahara, S. and Muguruma, M. 2009. Effect of microbial transglutaminase on the natural actomyosin cross-linking in chicken and beef. Meat Sci. 82: 170-178.

Aidaroos, H. I., Du, G. and Chen, J. 2011. Microbial fed-batch production of transglutaminase using ammonium sulphate and calcium chloride by *Streptomyces hygroscopicus*. Biotechnol. Bioinf. Bioeng. 1: 173-178.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Mille, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613-2617.

Ávila, M. D. R., Ordóñez, J. A., Hoz, L. l., Herrero, A. M. and Cambero M. I. 2010. Microbial transglutaminase for cold-set binding of unsalted/salted pork models and restructured dry ham. Meat Sci. 84: 747-754.

Auld, D. S. 2001. Zinc coordination sphere in biochemical zinc sites. Biometals. 14: 271–313.

Bahrim, G., Iancu, C., Butu, N. and Negoita, T. G. 2010. Production of a novel microbial transglutaminase using *Streptomyces* sp. polar strains. Romanian Biotechnol. Lett. 15: 5197-5203.

Baker, G. C., Smith, J. J. and Cowan, D. A. 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods. 55: 541-55.

Barros, S. L. H., Assmann, F. and Zachia, A. M. A. 2003. Purification and properties of a transglutaminase produced by *Bacillus circulans* strain isolated from the Amazon environment. Biotechnol. Appl. Biochem. 37: 295-299.

Barrow, G. I. and Feltham, R. K. A. 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria, 3rd edn. Cambridge University Press, Cambridge.

Bae, S. J., Kim, H. S., Hwang, Y. I. and Choi, S. U. 2012. Cloning of transglutaminase gene from *Streptomyces platensis* YK-2 and its high expression in *Streptomyces* strains. J. Korean Soc. Appl. Biol. Chem. 55: 417−422.

Bech, L., Norreveng, I. A., Halkier, T., Rasmussen, G., Schafer, T. and Andersen, J. T. 2001. Microbial transglutaminase, their production and use. US6190879B1.

Benjakul, S., Chantarasuwan, C. and Visessanguan, W. 2003. Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. Food Chem. 82: 567–574.

Benjakul, S. and Bauer, F. 2001. Biochemical and physicochemical changes in catfish (*Silurus glanis Linne*) muscle as influenced by different freeze–thaw cylces. Food Chem. 72: 207–217.

Biocompare. 2013. Bench Tips: Choosing the right DNA polymerase for PCR (Online). Available http://www.biocompare.com/Bench-Tips/146243-Choosing-the-Right-DNA-Polymerase-for-PCR/ (21 November 2018).

Blobel, G. and Sabatini, D. D. 1970. Controlled proteolysis of nascent polypeptides in rat liver cell fractions. I. Location of the polypeptides within ribosomes. J. Cell Biol. 45: 130-145.

Bourneow, C., Benjakul, S., Sumpavapol, S. and H-Kittikun, A. 2012a. Isolation and cultivation of transglutaminase-producing bacteria from seafood processing factories. Innov. Rom. Food Biotechnol. 10: 28-39.

Bourneow, C., Benjakul, S. and H-Kittikun, A. 2012. Hydroxamate-based colorimetric method for direct screening of transglutaminase-producing bacteria. World J. Microbiol. Biotechnol. 28: 2273-2277.

Bourneow, C. 2011. Microbial transglutaminase from bacteria isolated from seafood processing wastes and its applications. Ph. D. Thesis in Food Science and Technology. Prince of Songkla University.

Bourneow, C., Benjakul, S. and H-Kittikun, A. 2010. Purification and characterization of microbial transglutaminase from *Enterobacter* sp. C2361. *In* Proceedings of the International Conference on Agriculture and Agro-Industry (ICAAD2010) Food, Health and Trade, Thailand. 19-20 November 2010. p.1-7.

Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72: 248–254.

Brenner, D. J., McWhorter, A. C., Kai, A., Steigerwalt, A. G. and Famer, J. J. 1986. *Enterobacter asburiae* sp. nov., a new species found in clinical specimens, and reassignment of *Erwinia dissolvens* and *Erwinia nimipressuralis* to the genus *Enterobacter* as *Enterobacter dissolvens* comb. nov. and *Enterobacter nimipressuralis* comb. nov. J. Clin. Microbiol. 23: 1114–1120.

Brenner, D. J., Richard, C., Steigerwalt, A. G., Asbury, M. A. and Mandel, M. 1980. *Enterobacter gergoviae* sp. nov.: a new species of *Enterobacteriaceae*

found in clinical specimens and the environment. Int. J. Syst. Bacteriol. 30: 1-6.

Brenner, D. J. 1984. Facultatively anaerobic gram-negative rods. Family I. *Enterobacteriaceae*. *In* Bergey's Manual of Systematic Bacteriology. vol. 1. (Krieg, N. R. and Holt, J. G., ed.). Baltimore. Williams and Wilkins. p. 408-420.

Brenner, D. J., Farmer, J. J. I., Fanning, G. R., Steigerwalt, A. G., Klykken, P., Wathen, H. G., Hickman, F. W. and Ewing, W. H. 1978. Deoxyribonucleic acid relatedness of *Proteus* and *Providencia* species. Int. J. Syst. Bacteriol. 28: 269-282.

Browning, D. F. and Busby, S. J. W. 2004. The regulation of bacterial transcription initiation. Nat. Rev. Microbiol. 2: 57-65.

Butler, J. N. 1964. Ion equilibrium: A mathematical approach. Addison-Wesley. p. 151.

Buttkus, H. 1970. Accelerated denaturation of myosin in frozen solution. J. Food Sci. 32: 558-561.

Carpousis, A. J. and Gralla, J. D. 1980. Cycling of ribonucleic acid polymerase to produce oligonucleotides during initiation in vitro at the *lac* UV5 promoter. Biochemistry. 19: 3245-3253.

Cheng, L., Zhang, D.X., Du, G.C. and Chen, J. 2007. Improvement on the activity of microbial transglutaminase with *Streptomyces hygroscopicus* by the addition of surfactant CTAB. Chin. J. Biotechnol. 23: 497-501.

Choi, B. K., Wyss, C. and Gobel, U. B. 1996. Phylogenetic analysis of pathogen-related oral spirochetes. J. Clin. Microbiol. 34:1922-1925.

Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K., and Lim, Y. W. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on

16S ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57: 2259-2261.

Cui, L., Du, G., Zhang, D. and Chen, J. 2008. Thermal stability and conformational changes of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. Bioresour. Technol. 99: 3794-3800.

Cui, L., Du, G., Zhang, D., Liu, H. and Chen, J. 2007. Purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. Food Chem. 105: 612-618.

Cui, L., Zhang, D. X., Huang, L., Liu, H., Du, G. C. and Chen, J. 2006. Stabilization of a new microbial transglutaminase from *Streptomyces hygroscopicus* WSH03-13 by spray drying. Process Biochem. 41: 1427-1431.

Date, M., Yokoyama, K., Umezeawa, Y., Matsui, H. and Kikuchi, Y. 2004. High level expression of *Streptomyces mobaraensis* transglutaminase in *Corynebacterium glutamicum* using a chimeric pro-region from *Streptomyces cinnamoneus* transglutaminase. J. Biotechnol. 110: 219-226.

Date, M., Yokoyama, K. Umezawa, Y. Matsui, H. and Kikuchi, Y. 2003. Production of native-type *Streptoverticillium mobaraense* transglutaminase in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 65: 3011-3014.

Dondero, M., Figueroa, V., Morales, X. and Curotto, E. 2006. Transglutaminase effects on gelation capacity of thermally induced beef protein gels. Food Chem. 99: 546–554.

Dube, M. Schafer, C., Neidhart, S. and Carle, R. 2007. Texturisation and modification of vegetable proteins for food applications using microbial transglutaminase. Eur. Food Res. Technol. 225: 287-299.

Duran, D., Junque, M., Schmitter, J. M., Gancet, C. and Goulas, P. 1998. Purification characterization and gene cloning of transglutaminase from *Streptomyces cinnamoneum* CBS 683.68. Biochimie. 80: 313-319.

de Haseth, P. L., Zupancic, M. L. and Record, T. J. M. 1998. RNA polymerase-promoter interactions: the comings and going of RNA polymerase. J. Bacteriol. 180: 3019-3025.

Euzéby, J. P. 2018. List of prokaryotic names with standing in nomenclature (Online). Available http://www.bacterio.cict.fr/e/enterobacteriaceae.html (15 October 2018).

Euzéby, J. P. 2018. List of bacterial names with standing in nomenclature: a folder (Online). Available on the internet. http://www.bacterio.net (15 October 2018).

Ewing, W. H. and Fife, M. A. 1968. *Enterobacter hafniae*. Int. J. Syst. Bacteriol. 18: 263-271.

Ezaki, T., Hashimoto, Y. and Yabuuchi, E. 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternation to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39: 224-229.

Felsenstien, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39: 738-791.

Folk, J. E. and Finlayson, J. S. 1977. The ε-γ-(glutamyl) lysine crosslink and the catalytic role of transglutaminases. Adv. Protein Chem. 311-133.

Folk, J. E. and Cole, P. W. 1966. Mechanism of action of guinea pig liver transglutaminase. I. Purification and properties of the enzyme: identification of a functional cystein essential for activity. J. Biol. Chem. 241: 5518-5525.

Folk, J. K. and Cole, P. W. 1965. Structural requirements of specific substrates for guinea pig liver transglutaminase. J. Biol. Chem. 240: 2951-2960.

Forbes, L. 1981. Rapid flagella stain. J. Clin. Microbiol. 13: 807-809.

Fredrich, P. and Aszodi, A. 1992. Calcium-dependent proteolysis and isopeptide bond formation: calpains and transglutaminase. Pure Appl. Chem. 64: 1093-1097.

Freudl, R. 2018. Signal peptides for recombinant protein secretion in bacterial expression systems. Microb. Cell Fact. 17: 52-61.

Gauche, C., Tomazi, T., Barreto, P. L. M., Ogliari, P. J. and Luiz, B. M. T. 2009. Physical properties of yoghurt manufactured with milk whey and transglutaminase. LWT - Food Sci. Technol. 42: 239-243.

Gauche, C., Vieira, J. T. C., Ogliaria, P. J. and Luiz, M. T. B. 2008. Crosslinking of milk whey proteins by transglutaminase. Process Biochem. 43: 788-794.

Galisa, P. S., da Silva, H. A. P., Macedo, A. V. M., Reis, V. M., Vidale, M. S., Baldanie, J. I., Simões-Araújoe, J. L. 2012. Identification and validation of reference genes to study the gene expression in Gluconacetobacter diazotrophicus grown in different carbon sources using RT-qPCR. J. Microbiol. Methods. 91: 1-7.

GE Healthcare. 2010. Ni Sepharose™ 6 Fast Flow. Sweden.

Gil, R., Silva, F. J., Pereto, J. and Moya1, A. 2004. Determination of the core of a minimal bacterial gene set. Microbiol. Mol. Biol. Rev. 68: 518-537.

Glusker, J. P., Katz, A. K. and Bock, C. W. 1999. Metal ion in biological systems. J. Rigaku. 18: 8-16.

Gopal, G. J. and Kumar, A. 2013. Strategies for the production of recombinant protein in *Escherichia coli*. Protein J. 32: 419-425.

Gonçalves, A. A. and Passos, M. G. 2010. Restructured fish product from white croacker (*Micropogonias furnieri*) mince using microbial transglutaminase. Braz. Arch. Biol. Technol. 53: 987-995.

Greenberg, C. S., Birckbichler, P. J. and Rice, R. H. 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. 5: 3071-3077.

Gruber, T. M. and Gross, C. A. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. 57: 441-466.

Ha, C. R. and Iuchi, I. 1997. Purification and partial characterization of 76 kDa transglutaminase in the egg envelope (chorion) of rainbow trout, *Oncorhynchus mykiss*. J. Biochem. 122: 947-954.

Hermann, D. and Foernzler, D. 2002. Specific amplification of difficult PCR products from small amounts of DNA using faststart taq DNA polymerase. Biochemica. 4: 25-26.

Hickman-Brenner, F. W., Farmer, J. J. I., Steigerwalt, A. G. and Brenner, D. J. 1983. *Providencia rustigianii*: a new species in the family *Enterobacteriaceae* formerly known as *Providencia alcalifaciens* biogroup 3. J. Clin. Microbiol. 17: 1057-1060.

H-Kittikun, A., Bourneow, C. and Benjakul, S. 2012. Hydrolysis of surimi waste waster for production of transglutaminase by *Enterobacter* sp. C2361 and *Providencia* sp. C1112. Food Chem. 135: 1183-1191.

Ho, M. L., Leu, S. Z., Hsieh, J. F. and Jiang, S. T. 2000. Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from *Streptomyces ladakanum.* J. Food Sci. 65: 76-80.

Hoffmann, H., Stindl, S., Ludwig, W, Stumpf, A., Mehlen, A., Heesemann, J., Monget, D., Schleifer, K. H., and Roggenkamp, A. 2005. Reassignment of *Enterobacter dissolvens* to *Enterobacter cloacae* as *E. cloacae* subspecies *dissolvens* comb. nov. and emended description of *Enterobacter asburiae* and *Enterobacter kobei*. Syst. Appl. Microbiol. 28: 196-205.

Hong, K., Gao, A., Xie, Q., Gao, H., zhuang, L., Yu, H. and Ruan, M. J. 2009. Actinomycetes for marine drug discovery isolation from Mangrove soils and plants in china microbiological research. Mar. Drugs., 7: 24-44.

Hormaeche, E. and Edwards, P. R. 1960. A proposed genus *Enterobacter*. Int. Bull. Bacteriol. Nomencl. Taxon. 10: 71-74.

Hsu, L. M., Vo, N. V., Kane, C. M. and Chamberlin, M. J. 2003. *In vitro* studies of transcript initiation by *Escherichia coli* RNA polymerase. 1. RNA chain initiation, abortive initiation, and promoter escape at three bacteriophage promoters. Biochem. 42: 3777-3786.

Hucker, G. L. and Conn, H. J. 1923. Method of Gram staining. Tech. Bull. N. Y. St. Agric. Exp. Sta. 93: 3-37.

Ichinose, A., Bottenus, P. J. and Davie, E. W. 1990. Structure of transglutaminase. J. Biol. Chem. 265: 13411-13414.

Itaya, H. and Kikuchi, Y. 2008. Secretion of Streptomyces mobaraensis pro-transglutaminase by coryneform bacteria. Appl. Microbiol. Biotechnol. 78: 621-625.

Janda, J. M. and Abbott, S. L. 2006. The Enterobacteria, 2nd ed. Washington, DC: American Society for Microbiology.

Jiang, S. T. and Lee, J. J. 1992. Purification characterization and utilization of pig plasma factor XIIIa. J. Agri. Food Chem. 40: 1101-1107.

Josten, A., Haalck, L., Spener, F. and Meusel, M. 2000. Use of microbial transglutaminase for the enzymatic biotinylation of antibodies. J. Immunol. Meth. 240: 47-54.

Juneja, P., and Lazzaro, B. P. 2009. *Providencia sneebia* sp. nov. and *Providencia burhodogranariea* sp. nov., isolated from wild *Drosophilla melanogaster*. Int J Syst Evol Microbiol. 59: 1108-1111.

Kampfer, P., Ruppel, S. and Remus, R. 2005. *Enterobacter radicincitans* sp. nov., a plant growth promoting species of the family *Enterobacteriaceae*. Syst. Appl. Microbiol. 28: 213-221.

Kanaji, T., Ozaki, H., Takao, T., Kawajiri, H., Ide, H., Motoki, M. and Shimonishi, Y. 1993. Primary structure of microbial transglutaminase from *Streptomyces* sp. Strain s-8112. J. Biol. Chem. 268 : 11565-11572.

Karami, M., Ebrahimzadeh, M. A., Mahdavi, M. R., Ghasami, M. and Salarian, A. 2009. Determination of effect of Iron (Fe^{2+} and Fe^{3+}) on human erythrocyte AChE activity. Asian J. Chem. 21: 2461-2467.

Kashiwagi, T. Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H. and Suzuki, E. 2002. Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. J. Biol. Chem. 277: 44252- 44260.

Kawai, M., Takehana, S. and Takagi, H. 1997. High-level expression of the chemically synthesized gene for microbial transglutaminase from *Streptomyces* in *Escherichia coli.* Biosci. Biotechnol. Biochem. 61(5): 830-835.

Kavitha, A., Vijayalakshmi, M., Sudhakar, P. and Narasimha, G. 2010. Screening of actinomycete strains for the production of antifungal metabolite. Afr. J. Microbiol. Res. 4: 27-32.

Kieliszek, M. and Misiewicz, A. 2014. Microbial transglutaminase and its application in the food industry. A review. Folia Microbiol. 59:241-250.

Kikuchi, Y., Date, M., Umezawa, Y., Yokoyama, K. and Matsui, H. 2011. Process for producing transglutaminase. US7972829B2.

Kikuchi, Y., Date, M., Yokoyama, K., Umezawa, Y. and Matsui, H. 2003. Secretion of active-form *Streptomyces mobaraense* transglutaminase by *Corynebacterium glutamicum:* processing of the pro-transglutaminase by a

cosecreted subtilisin-like protease from *Streptomyces albogriseolus*. Appl. Environ. Microbiol. 69: 358-366.

Kim, S. B., Falconer, C., Williams, E. & Goodfellow, M. 1998. *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soil. Int. J. Syst. Bacteriol. 48: 59-68.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparation studies of nucleotide sequence. J. Mol. Evol. 16: 111-120.

Klein, J. D., Guzman, E. and Kuehn, G. D. 1992. Purification and partial characterization of transglutaminase from *Physarum polycephalum*. J. Bacteriol. 174: 1599-2605.

Kobayashi, K., Hashiguchi, K. I., Yokozeki, K. and Yamanaka, S. 1998. Molecular cloning of the transglutaminase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. Biosci. Biotechnol. Biochem. 62: 1109-1114.

Kobayashi, K., Kumazawa, Y., Miwa, K. and Yamanaka, S. 1996. ε-(γ-Glutamyl) lysine cross-links of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. FEMS Microbiol. Lett. 144: 157-160.

Krummel, B. and Chamberlin, M. J. 1989. RNA chain initiation by *Escherichia coli* RNA polymerase. Structural transitions of the enzyme in early ternary complexes. Biochemistry. 28: 7829-7842.

Kumazawa, Y., Nakanishi, K., Yasueda, H. and Motoki, M. 1996. Purification and characterization of transglutaminase from walleye pollack liver. Fisheries Sci. 62: 959-964.

Kumazawa, Y., Numazawa, T., Seguro, K. and Motoki, M. 1995. Suppression of surimi gel setting by transglutaminase inhibitors. J. Food Sci. 60: 715-717.

Kuraishi, C., Sakamoto, J. and Soeda, T. 1996. The usefulness of transglutaminase for food processing. *In* Biotechnology for Improved Food and Flavors, ACS symposium series 637. (Takeoka G. R., Teranishi, R., Williams, P. J. and Kobayashi, A., ed.) American Chemical Society. Washington, DC. P. 29-38.

Laemmli, U. K. 1970. Cleavage of structure proteins during assembly of head bacteriophage T4. Nature. 277: 680-685.

Lee, J. J., Tzeng, S. S., Wu, J. and Jiang, S. T. 2000. Inhibition of thermal degradation of mackerel surimi by pig plasma protein and L-kininogen. J. Food Sci. 65: 1124-1129.

Li, H., Zhang, L., Cui, Y., Luo, X., Xue, C. and Wang, S. 2013. Expression of soluble recombinant transglutaminase from *Zea mays* in *Pichia pastoris*. World J. Microbiol. Biotechnol. 1-9.

Lin, S. J., Hsieh, Y. F., Lai, L. A., Chao, M. L., and Chu, W. S. 2008. Characterization and large-scale production of recombinant *Streptoverticillium platensis* transglutaminase. J. Ind. Microbiol. Biotechnol. 35: 981-990.

Lin, S. J., Hsieh, Y. F., Wang, P. M. and Chu, W. S. 2007. Efficient purification of transglutaminase from recombinant *Streptomyces platensis* at various scales. Biotechnol. Lett. 29: 111-115.

Lin, Y. S., Chao, M. I., Liu, C. H., Tseng, M. and Chu, W. S. 2006. Cloning of the gene coding for transglutaminase from *Streptomyces platensis* and its expression in *Streptomyces lividans*. Process Biochem. 41: 519-524.

Lin, Y. S., Chao, M. L., Liu, C. H. and Chu, W. S. 2004. Cloning and expression of the transglutaminase gene from *Streptomyces ladakanum* in *Streptomyces lividans*. Process Biochem. 80: 591-598.

Liu, S., Wan, D., Wang, M. Madzak, C., Du, G. and Chen, J. 2015. Overproduction of pro-transglutaminase from *Streptomyces hygroscopicus* in *Yarrowia lipolytica* and its biochemical characterization. BMC Biotechnol. 15: 1-9.

Liu, S., Zhang, D., Wang, M., Cui, W., Chen, K., Liu, Y., Du, G., Chen, J. and Zhou, Z. 2011a. The pro-region of *Streptomyces hygroscopicus* transglutaminase affects its secretion by *Escherichia coli*. FEMS Microbiol. Lett. 324: 98-105.

Liu, S., Zhang, D., Wang, M., Cui, W., Chen, K., Du, G., Chen, J. and Zho, Z. 2011b. The order of expression is a key factor in the production of active transglutaminase in *Escherichia coli* by co-expression with its pro-peptide. Microb. Cell Fact. 10:112-118.

Liu, X. Q., Yang, X. Q., Xie, F. H., Song, L. Y., Zhang, G. Q. and Qian, S. J. 2007. On-column refolding and purification of transglutaminase from *Streptomyces fradiae* expressed as inclusion bodies in *Escherichia coli*. Protein Expr. Purif. 51: 179-186.

Liu, X., Yang, X., Xie, F. and Qian, S. 2006. Cloning of transglutaminase gene from *Streptomyces fradiae* and its enhanced expression in the original strain. Biotechnol. Lett. 28: 1319-1325.

Lorand, L. and Graham, R. M. 2003. Transglutaminase, cross-linking enzymes with pleiotropic function. Mol. Cell Biol. 4: 140-156.

Lu, S. Y., Zhou, N. D., Tian, Y. P. and Chen, J. 2003. Purification and properties of transglutaminase from *Streptomyces mobaraensis*. J. Food Biochem. 27: 109-126.

Lu, J. J., Perng, C. L., Lee, S. Y. and Wan, C. C. 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. J. Clin. Microbiol. 38: 2076-2080.

Luciano, F. B. and Arntfield, S. D. 2012. Use of transglutaminases in foods and potential utilization of plants as a transglutaminase source-review. Biotemas. 25: 1-11.

Luis, S. J. T., Cabriales, J. J. G., Ramírez, J. A. and Vázquez, M. 2004. Production of transglutaminase by *Streptomyces ladakanum* NRRL-3191 grown on media made from hydrolysates of Sorghum Straw. Technol. Biotechnol. 42: 1-4.

Macedo, J. A., Sette, L. D. and Sato, H. H. 2011. Purification and characterization of a new transglutaminase from *Streptomyces* sp. in Brazilian soil. J. Food Biochem. 35: 1361-1372.

Macedo, J. A., Sette, L. D. and Sato, H. H. 2010. A comparative biochemical characterization of microbial transglutaminases: commercial vs. a newly isolated enzyme from *Streptomyces* sp. Food Bioprocess Tech. 3: 308–314.

Macedo, J. A., Sette, L. D. and Sato, H. H. 2007. Optimization of medium composition for transglutaminase production by a Brazilian soil *Streptomyces* sp. Electr. J. Biotechnol. 10: 618–626.

Madhaiyan, M., Poonguzhali, S., Lee, J. S., Saravanan, V. S., Lee, K. C. and Santhanakrishnan, P. 2010. *Enterobacter arachidis* sp. nov. a plant-growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut. Int. J. Syst. Evol. Microbiol. 60: 1559-1564.

Makarova, K., Aravind, L. and Koonin, E. V. 1999. A superfamily of archaeal, bacterial and eukaryotic proteins homologous to animal transglutaminases. Prot. Sci. 8: 1714-1719.

Manos, J. and Belasb , R. 2006. The Genera *Proteus*, *Providencia*, and *Morganella*. *In* The Prokaryotes: Proteobacteria: Gamma Subclass. Vol. 6. 3rd. (Falkow, S., Rosenberg, E., Schleifer, K. H., Stackebrandt, E. and Dworkin, M., ed.). Springer. New York. p. 245-269.

Manter, D. K., Hunter, W. J. and Vivanco, J. M. 2011. *Enterobacter soli* sp. nov.: A Lignin-degrading γ-proteobacteria isolated from soil. Curr. Microbiol. 62: 1044-1049.

Marx, C. K., Hertel, T. C. and Pietzsch, M. 2008. Purification and activation of a recombinant histidine-tagged pro-transglutaminase after soluble expression in *Escherichia coli* and partial characterization of the active enzyme. Enzyme Microb. Tech. 42: 568-575.

Marx, C. K., Hertel, T. C. and Pietzsch, M. 2007. Soluble expression of a pro-transglutaminase from *Streptomyces mobaraensis* in *Escherichia coli*. Enzyme Microb. Tech. 40: 1543-1550.

McCabe, K. M., Zhang, Y. H., Huang, B. L., Wagar, E. A. and McCabe, E. R. 1999. Bacterial species identification after DNA amplification with a universal primer pair. Mol. Genet. Metab. 66: 205-211.

Mea, M. D., Ruiz, C. D., Claparols, I., Fracassini. S. D. and Rigau, J. 2004. The first plant transglutaminase. Plant Physiol. 135: 2046-2054.

Meiying, Z., Guocheng, D. and Jian, C. 2002 pH control strategy of batch microbial transglutaminase production with *Streptomyces mobaraense*. Enzyme Microb. Tech. 31: 477-481.

Motoki, M. and Seguro, K. 1998. Transglutaminase and its use for food processing. Trends Food Sci. Technol. 9: 204-210.

Munson, M. A., Banerjee, A., Watson, T. F. and Wade, W. G. 2004. Molecular analysis of the microflora associated with dental caries. J. Clin. Microbiol. 42: 3023-3029.

Muller, H. E., O'Hara, C. M., Fanning, G. R., Hickman-Brenner, F. W., Swenson, J. M., and Brenner, D. J. 1986. *Providencia heimbachae*, a new species of *Enterobacteriaceae* isolated from animals. Int. J. Syst. Bacteriol. 36: 252-256.

Nazawa, H., Mori, T., Kimura, M. and Seki, N. 2005. Characterization of a transglutaminase from scallop hemocyte and identification of its intracellular substrates. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 140: 395-402.

Negus, S. S. 2001. A novel microbial transglutaminase derived from *Streptomyces baldaccii*. Ph. D. Biomolecular and Biomedical Science. Griffith University, Nathan Campus Queensland, Australia.

Neilson, P. M. 1995. Reaction and potential industrial application of transglutaminase. Review of literature and patents. Food Biotechnol. 9: 119-156.

Nielsen, J. E. and Mccammon, A. J. 2003. Calculating pKa values in enzyme active sites. Protein Sci. 12: 1894-1901.

Noda, S., Miyazaki, T., Tanaka, T., Ogino, C. and Kondo, A. 2012. Production of *Streptomyces cinnamoneum* transglutaminase and cinnamic acid by recombinant *Streptomyces lividans* cultured on biomass-derived carbon sources. Bioresour. Technol.104: 648-651.

Noda, S., Ito, Y., Shimizu, N., Tanaka, T., Ogino, C. and Kondo, A. 2010. Over-production of various secretory-form proteins in *Streptomyces lividans*. Protein Expr. Purif. 73: 198-202.

Noguchi, K., Ishikawa, K., Yokoyama, K., Ohtsuka, T., Nio, N. and Suzuki, E. 2001. Crystal structure of red sea bream transglutaminase. J. Biol. Chem. 276: 12055-12059.

Novagen. 2003. pET System Manual, 10th ed. United States.

Nozawa, H., Mamegoshi, S., and Seki, N. 1997. Partial purification and characterization of six transglutaminase from ordinary muscles of various fishes and marine invertebrates. Biochem. Physiol. B. 118: 313-317.

O'Hara, C. M., Brenner, F. W. and Miller, J. M. 2000. Classification, identification and clinical significance of *Proteus, Providencia* and *Morganella*. Clin. Microbiol. Rev. 13: 534-546.

Ohtsuka, T., Sawa, A., Kawabata, R., Nio, N. and Motoki, M. 2000. Substrate specificities of microbial transglutaminase for primary amines. J. Agric. Food Chem. 48: 6230-6233.

Owen, R. J., Ahmed, A. U., and Dawson, C. A. 1987. Guanine-plus-cytosine contents of type strains of the genus *Providencia*. Int. J. Syst. Bacteriol. 37: 449-450.

Pace, N. J. and Weerapana, E. 2014. Zinc-binding cysteines: diverse functions and structural motifs. Biomolecules. 4: 419-434.

Pasternack, R. Dorsch, S. Otterbach, J. T., Robenek, I. R. and Fuchsbauer, H. L. 1998. Bacterial pro-transglutaminase from *Streptoverticillium mobaraense* purification, characterisation and sequence of the zymogen. Eur. J. Biochem. 257: 570-576.

Pedersen, L. C., Yee, V. C., Bishop, P. D., Le T. I., Teller, D. C. and Stenkamp, R. E. 1994. Transglutaminase factor XIII uses proteinase-like catalytic triad to crosslink macromolecules. Protein Sci. 3: 1131-1135.

Peer, V. Y., Chapelle, S. and De Wachter, R. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. 24: 3381-3391.

Penner, J. L. and Hennessy, J. N. 1979. Application of O-serotyping in a study of *Providencia rettgeri* (*Proteus rettgeri*) isolated from human and nonhuman sources. J. Clin. Microbiol. 10: 834-840.

Petti, C. A., Polage, C. R. and Schreckenberger, P. 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. J. Clin. Microbiol. 43:6123-6125.

Polster, M., and Svobodova, M. 1964. Production of reddish-brown pigment from DL-tryptophan by enterobacteria of the *Proteus-Providencia* group. Experientia. 20: 637-638.

Rachel, N. M. and Pelletier, J. N. 2013. Biotechnological applications of transglutaminases. Biomolecules. 3: 870-888.

Robertson, D., Shore, S. and Miller, D. M. 1997. Manipulation and expression of recombinant DNA: A laboratory manual. Academic Press, New York.

Robichon, C., Luo, J., Causey, T. B., Benner, J. S. and Samuelson, J. C. 2011. Engineering *Escherichia coli* BL21 (DE3) derivative strains to minimize *E. coli* protein contamination after purification by immobilized metal affinity chromatography. Appl. Environ. Microbiol. 77: 4634-4646.

Rossa, P. N., Sá, E. M. F., Burin, V. M. and Luiz, M. T. B. 2011. Optimization of microbial transglutaminase activity in icecream using response surface methodology. LWT-J. Food Sci. Tech. 44: 29-34.

Ruiz-Garcia, C., Bejar, V., Martinez-Checa, F., Llamas, I. and Quesada, E. 2005. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Velez in Malaga, southern Spain. Int. J. Syst. Evol. Microbiol. 55: 191-195.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Saito, H. and Miura, K. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta. 72: 619-629.

Sakamoto, H. 1996. Strength enhancement by addition of microbial transglutaminase during Chinese noodle processing. Nippon Shokuhin Kagaku Kaishi. 43: 598-602.

Sakamoto, S., Kumazawa, Y., Kawajiri, H. and Motoki, M. 1995. ε-(γ-glutaminyl) lysine crosslink distribution in foods as determined by improved method. J. Food Sci. 60: 416-419.

Sakamoto, H., Nonaka, M. and Motoki, M. 1993. Calcium-independent transglutaminase derived from a microorganism: its characteristic and capability in protein crosslinking and gel formation. Food Hydrocoll. 8: 383-386.

Samelak, A., Sobieszczuk-Nowicka, E. and Legocka, J. 2010. Transglutaminazy i ich biologiczne funkcje. Postepy Biol. Komorki. 37:599-612.

Sanders, W. E. and Sanders, C. C. 1997. *Enterobacter* sp.: a pathogen poised to flourish at the turn of century. Clin. Microbiol. Rev. 10: 220-241.

Santos, M. and Torné, J. M. 2009. Recent patents on transglutaminase production and applications: A brief review. Recent Pat. Biotechnol. 3: 166-174.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids, MIDI technical note 101, Microbial ID, Inc., Newark, DE.

Schmalenberger, A., Schwieger, F., Tebbe, C. C. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. 67: 3557-3563.

Schroder, E., Phillips, C., Garman, E., Harlos, K. and Crawford, C. 1993. X-ray crystallographic structure of papain-leupeptin complex. FEBS Lett. 315: 38-42.

Schumann, W. and Ferreira, L. C. S. 2004. Production of recombinant proteins in *Escherichia coli*. Gen. Mol. Biol. 27: 442-453.

Seguro, K., Nio, N. and Motoki, M. 1996. Some characteristics of a microbial protein cross-link enzyme: transglutaminase in macromolecular. Inter. Food Technol. American Chemical Society: Washington, DC. p. 271-280.

Seki, N., Uno, H., Lee, N. H., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in alaska pollack muscle and surimi and its reaction with myosin B. Nippon Suisan Gakkaishi. 56: 125-132.

Seledtsov, I. A., Vul'f, I. and Makarova, K. S. 1995. Multiple alignment of biopolymer sequences, based on the search for statistically significant common segments. Mol. Biol. (Mosk) 29: 1023-1039.

Seravalli, E. A. G., Igutia, A. M., Santanaa, I. A. Filho, F. F. 2011. Effects of application of transglutaminase in wheat proteins during the production of bread. Procedia Food Sci. 1: 935-942.

Shida, O., Takagi, H., Kadowaki, K., and Komagata, K. 1996. Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int. J. Syst. Bacteriol. 46: 939-946.

Shimba, N., Yokoyama, K. and Suzuki, E. 2002. NMR-based screening method for transglutaminases: Rapid analysis of their substrate speci-ficities and reaction rates. J. Agric. Food Chem. 50: 1330-1334.

Shleĭkin, A. G. and Danilov, N. P. 2011. Evolutionary-biological peculiarities of transglutaminase. Structure, physiological functions, application. Zh. Evol. Biokhim. Fiziol. 47: 3-14.

Somvanshi, V. S., Lang, E., Staubler, B., Sproer, C., Schumann, P., Ganguly, S., Saxena, A. K., and Stackebrandt, E. 2006. *Providencia vermicola* sp. nov., isolated from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*. Int. J. Syst. Evol. Microbiol. 56: 629-633.

Sørensen, H. P. and Mortensen, K. K. 2005. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb. Cell Fact. 4: 1-8.

Souza, C. F. V., Flores, S. H. and Ayub, M. Z. 2006. Optimization of medium composition for the production of transglutaminase by *Bacillus circula*ns

BL32 using statistical experimental methods. Process Biochem. 41: 1186-1192.

Stephan, R., Trappen, S. V., Cleenwerck, I., Iversen, C., Joosten, H., Vos, P. D. and Lehner, A. 2008. *Enterobacter pulveris* sp. nov., isolated from fruit powder, infant formula and an infant formula production environment. Int. J. Syst. Evol. Microbiol. 58: 237-241.

Stephan, R., Trappen, S. V., Cleenwerck, I., Iversen, C., Joosten, H., Vos, P. D. and Lehner, A. 2008. *Enterobacter pulveris* sp. nov., isolated from fruit powder, infant formula and an infant formula production environment. Int. J. Syst. Evol. Microbiol. 58: 237-241.

Stephan, R., Trappen, S. V., Cleenwerck, I., Vancanneyt, M., Vos, P. D. and Lehner, A. 2007. *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov., isolated from fruit powder. Int. J. Syst. Evol. Microbiol. 57: 820-826.

Studier, W. F. 2005. Protein production by auto-induction in high-density shaking cultures. Protein Expr. Purif. 41: 207–234.

Susanti, E. V. H. 2003. Isolation and characterization of protease from *Bacillus subtilis* 1012M15 Biodiversitas. 4: 12-17.

Suzuki, S., Izawa, Y., Kobayashi, K., Eto, Y., Yamanaka, S., Kubota, K. and Yokozeki, K. 2000. Purification and characterization of novel transglutaminas from *Bacillus subtilis* spores. Biosci. Biotech. Biochem. 64: 2344-2351.

Suzuki, T., Maruyama, M. and Morohashi, M. 1995. Distribution of transglutaminase in quail uropygial glands. J. Vet. Med. Sci. 57: 55-58.

Taguchi, S., Arakawa, K., Yokoyama, K., Takehana, S., Takagi, H. and Momose, H. 2002. Overexpression and purification of microbial pro-transglutaminase from *Streptomyces cinnamoneum* and in vitro processing by *Streptomyces albogriseolus* proteases. J. Biosci. Bioeng. 94: 478-481.

Tamames, J. 2001. Evolution of gene order conservation in prokaryotes. Genome Biol. 2: 1-11.

Tamaoka, J. and Komagata, K. 1984. Determination of DNA base composition by reversed-phase high performance liquid chromatography. FEMS. Microbiol. Lett. 25: 125-128.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28: 2731-2739.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software Version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Téllez-Luis1, S. J., González-Cabriales, J. J., Ramírez, J. A. and Vázquez, M. 2004. Production of transglutaminase by *Streptomyces ladakanum* NRRL-3191 grown on media made from hydrolysates of sorghum straw. Food Technol. Biotechnol. 42: 1-4.

Thompson, J. D., Gibson, T. J., Plewniak, K., Jeanmougin, F. and Higgins, D. G. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acids. Res. 25: 4876-4882.

Tokunaga, F., Yamada, M., Miyata, T., Ding, Y. L., Kawabata, H. M., Muta.T., Iwanaga, S., Ichinose, A. and Davie, E. W. 1993. Limulus hemocyte transglutaminase. Its purification and characterization, and identification of the intracellular substrates. J. Biol. Chem. 268: 252-261.

Tomsic, M., Tsujikawa, L., Panaghie, G., Wang, Y., Azok, J. and de Haseth, P. L. 2001. Different roles for basic and aromatic amino acids in conserved region 2 of *Escherichia coli* sigma70 in the nucleation and maintenance of

the signle-stranded DNA bubble in open RNA polymerase-promoter complexes. J. Biol. Chem. 276: 31891-31896.

Trespalacios, P. and Pla, R. 2007. Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. Food Chem. 100: 264-272.

Tsai, G. J., Lin S. M. and Jaing, S. T. 1996. Transglutaminase from *Streptomyces ladakanum* and application to mince fish product. J. Food Sci. 61: 1234-1238.

Tseng, C. S. and Lai, H. M. 2002. Physicochemical properties of wheat flour dough modified by microbial transglutaminase. J. Food Sci. 67: 750-755.

Tsirigotaki, A., De Geyter, J. Sostaric. N., Economou, A. and Karamanou, S. 2017. Protein export through the bacterial Sec pathway. 15: 21-36.

Tsujikawa, L., Tsodikov, O. V. and de Haseth, P. L. 2002. Interaction of RNA polymerase with forked DNA: evidence for two kinetically significant intermediates on the pathway to the final complex. Proc. Nat. Acad. Sci. USA. 99: 3493-3498.

Truong, V. D., Clare, D. A., Catignani, G. and Swaisgood, H. E. 2004. Cross-linking and rheological changes of whey proteins treated with microbial transglutaminase. J. Agric. Food Chem. 52: 1170-1176.

Tzeng, S. S. ,Yin, L. J., Chen, G. H. and Jiang, S. T. 2005. Molecular cloning of microbial transglutaminase from *Streptoverticillium ladakanum* BCRC12422. J. Fish. Soc. 32: 53-67.

Villalobos, E., Santos, M., Talavera, D., Rodriguez-Falcon, M. and Torne, J. M. 2004. Molecular cloning and characterization of a maize transglutaminase complementary DNA. Gene. 336: 93-104.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsura, A., Takagi, H., Motoki, M. and Takeuchi, K. 1994. Molecular cloning of the gene for microbial

transglutaminase from *Streptomyces* and its expression in *Streptomyces lividans*. Biosci. Biotech. Biochem. 58: 82-87.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. and Truper, H. G. 1987. International Committee on Systematic Bacteriology. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37: 463-464.

Willemse, J., Borst, J., Waal, E., Bisseling, T. and Wezel, G. 2011. Positive control of cell division: FtsZ is recruited by SsgB during sporulation of *Streptomyces*. Genes Dev. 25: 89 -99.

Wootton, J. C. and Federhen, S. 1996. Analysis of compositionally biased regions in sequence databases. Methods Enzymol. 266: 554-571.

Wootton, J. C. 1994. Non-globular domains in protein sequences: automated segmentation using complexity measures. Comput. Chem. 18: 269–285.

Wu, J. W., Tsai, G. J. and Jiang, S. T., 1996. Screening the microorganism and some factors for 191 the production of transglutaminase. J. Chin. Agric. Chem. Soc. 34: 228-240.

Xie, L. and Wang, D. I. C. 1994. Applications of improved stoichiometric model in medium design and fed-batch cultivation of animal cells in bioreactor. Biotechnol. Bioeng. 43: 1164-1174.

Yan, G. L., Du, G. C., Li, Y., Chen, J. and Zhong, J. J. 2005. Enhancement of microbial transglutaminase production by *Streptomyces mobaraensis*: application of a two-stage agitation speed control strategy. Process Biochem. 40: 963-968.

Yang, H. L., Pan, L. and Lin, Y. 2009. Purification and on-column activation of a recombinant histidine-tagged protransglutaminase after soluble expression in *Escherichia coli*. Biosci. Biotechnol. Biotechem. 73: 2531-2534.

Yee, V. C., Pedersen, L. C., Le, T. I., Bishop, P. D., Stenkamp, R. E. and Teller, D. C. 1994. Three-dimensional structure of a transglutaminase: Human blood coagulation factor XIII. Proc. Natl. Acad. Sci. 91: 7296-7300.

Yeo, S. H., Yoon J. H., Lee, D. G. and Kim, H. S. 2009. Screening and identification of a *Streptomyces platensis* YK-2, a new transglutaminase producer. J. Microbiol. Biotechnol. 19: 588-595.

Yoh, M., Matsuyama, J., Ohnishi, M., Takagi, K., Miyagi, H., Mori, K., Park, K., Ono, T. and Honda, T. 2005. Importance of *Providencia* species as a major cause of travellers diarrhoea. J. Med. Microbiol. 54: 1077-1082.

Yokoyama, K., Utsumi, H., Nakamura, T., Ogaya, D., Shimba, N., Suzuki, E. and Taguchi, S. 2010. Screening for improved activity of a transglutaminase from *Streptomyces mobarensis* created by a novel rational mutagenesis and random mutagenesis. Appl. Microbiol. Biotechnol. 87: 2087-2096.

Yokoyama, K., Nio, N. and Kikuchi, Y. 2004. Properties and applications of microbial transglutaminase. Appl. Microbiol. Biotechnol. 64: 447-454.

Yokoyama, K. I., Nakamura, N., Seguro, K. and Kubota, K. 2000. Overproduction of microbial transglutaminase in *Escherichia coli*, in vitro refolding, and characterization of the refolded form. Biosci. Biotechnol. Biochem. 64: 1263-1270.

Yu, Y., Tang, B., Dai, R., Zhang, B., Chen, L., Yang, H., Zhao,G. and Ding, X. 2018. Identification of the streptothricin and tunicamycin biosynthetic gene clusters by genome mining in *Streptomyces* sp. strain fd1-xmd. Appl. Microbiol. Biotechnol. 102: 2621–263.

Yu, Y. J., Wu, S. C., Chan, H. H., Chen, Y. C., Chen, Z. Y. and Yang, M. T. 2008. Overproduction of soluble recombinant transglutaminase from *Streptomyces netropsis* in *Escherichia coli*. Appl. Microbiol. Biotechnol. 81: 523-32.

Yurimoto, H., Yamane, M., Kikuchi, Y., Matsui, H., Kato, N. and Sakai, Y. 2004. The pro-peptide of *Streptomyces mobaraensis* transglutaminase function in cis and in trans to mediate efficient secretion of active enzyme from methylotrophic yeasts. Biosci. Biotechnol. Biochem. 68: 2054-2069.

Zhang, D., Zhu, Y. and Chen, J. 2009. Microbial transglutaminase production: understanding the mechanism. Biotechnol. Genet. Eng. Rev. 26: 205-222.

Zhang, D., Wang, M., Wu, J., Cui, L., Du, G. and Chen, J. 2008. Two different proteases from *Streptomyces hygroscopicus* are involved in transglutaminase activation. J. Agric. Food Chem. 56, 10261-10264.

Zheng, M. Du, G. and Chen. J. 2002. pH control strategy of batch microbial transglutaminase production with *Streptomyces mobaraense*. Enzyme Microb. Tech. 31: 477-481.

Zheng, M., Du, G., Guo, W. and Chen. J. 2001. A temperature-shift strategy in batch microbial transglutaminase fermentation. Process Biochem. 36: 525-530.

Zilda, D. S. 2013. Purification and characterization of thermostable protease produced by *Brevibacillus* sp. LII isolated from Padangcermin hotspring Lampung. Gadjah Mada University.

Zotzel, J., Keller, P. and Fuchsbauer, H. L. 2003. Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease. Eur. J. Biochem. 270: 3214-3222.

Zhu, Y. and Tramper, J. 2008. Novel applications for microbial transglutaminase beyond food processing. Trends Biotechnol. 26: 559-565.

Zhu, Y., Rinzema, A., Tramper, J. and Bol, J. 1996. Medium design based on stoichiometric analysis of microbial transglutaminase production by *Streptomyces mobaraensis*. Biotechnol. Bioeng. 50: 291-298.

APPENDIX

1. Standard curve of enzyme

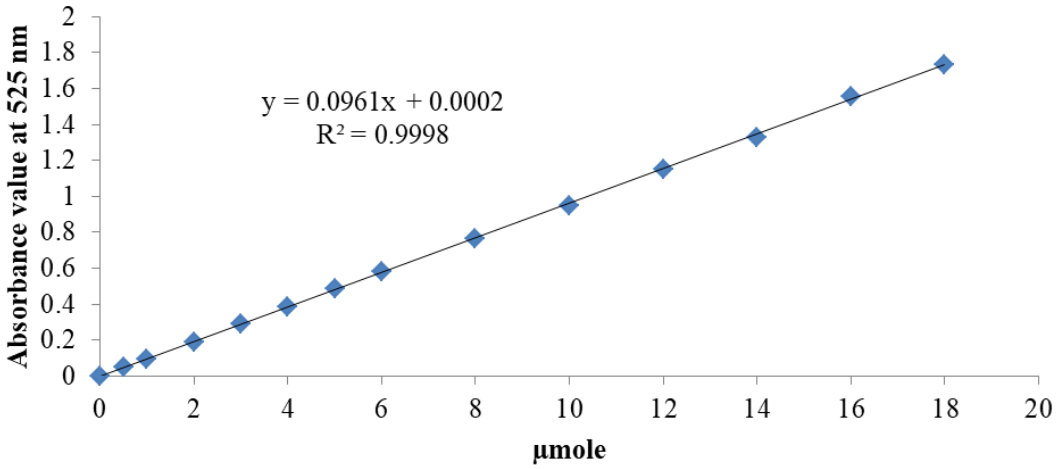


Figure 1 Calibration curve of Glutamic acid concentration by absorbance measurements at 525 nm.

VITAE

Name Miss Suwannee Khunthongpan

Student ID 6011030013

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (General Science)	Prince of Songkla University	2004
Master of Science (Biotechnology)	Prince of Songkla University	2009

Scholarships award during enrolment

1. The Japan Student Services Organization (JASSO)
2. The National Research University Project of Thailand's Office of the Higher Education Commission

List of publications and presentation

Publications

Khunthongpan, S., H-Kittikun, A., Bourneow, C., Tanasupawat, S., Benjakul, S. and Sumpavapol, P. 2013. *Enterobacter siamensis* sp. nov., a transglutaminase-producing bacterium isolated from seafood processing wastewater in Thailand. J. Gen. Appl. Microbiol. 59: 135-140

Khunthongpan, S., Sumpavapol, P., Tanasupawat, S., Benjakul, S. and H-Kittikun, A. 2013. *Providencia thailandensis* sp. nov., isolated from seafood processing wastewater. J. Gen. Appl. Microbiol. 59: 185-190.

Proceeding

Khunthongpan, S., H-Kittikun, A., Bourneow, C., Tanasupawat, S and Sumpavapol, P. 2012. Identification of transglutaminase-producing bacterium isolated from seafood processing wastewater. The proceedings of 2012 International

Conference on Nutrition and Food Science (ICNFS 2012), July 23-24, 2012, Singapore. (Oral presentation).